From the Department of Histology, University of Uppsala,
Uppsala, Sweden

STUDIES ON THE MIGRATION OF CELLS FROM GUT-ASSOCIATED LYMPHATIC TISSUE IN THE YOUNG RABBIT

by
Torsten E. R. Brenning

Uppsala 1975

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AKADEMISK AVHANDLING

som för avläggande av medicine doktors-examen vid Uppsala universitets medicinska fakultet kommer att offentligt försvaras i föreläsningssal B 21, Biomedicinska Centrum, Uppsala, fredagen den 30 maj 1975 kl. 09.15

av

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Uppsala 1975

Doctoral thesis at Uppsala University 1975

ISBN

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S-751 23 UPPSALA, Sweden

Printed in Sweden by Reklam & Katalogtryck
Uppsala 1975

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To my wife Gunilla

INTRODUCTION

LYMPHATIC TISSUES AND CELL MIGRATIONS

The immune response of mammals and birds is functionally divided into a cell mediated and antibody mediated component. The thymus regulates the ontogeny of the T lymphocytes, effector cells in cell-mediated immunity, whereas in birds the bursa of Fabricius is important for the normal development of B lymphocytes, antibody producing effector cells in antibody-mediated immunity (17,19,37,42,56,85,93,122). These two types of effector cell systems thus display a delineation in terms of ontogeny and certain functions, but may also interact in immune responses (14,24,84). Thus T lymphocytes can both act as "helpers" (59,60,82,86) and "suppressors" (38,59) of B lymphocyte antibody production.

In terms of ontogeny and function, the lymphatic organs can be divided into central lymphatic organs, i.e. the thymus and the avian bursa of Fabricius and peripheral lymphatic organs, e.g. the spleen and lymph nodes (78, 85).

The thymus and bursa of Fabricius seed lymphocytes to peripheral lymphatic organs (30,74,103). This traffic of cells is of importance for the maturation of the thymus- and bursa-dependent systems, but it does not preclude that the central lymphatic organs may exert a regulatory influence on peripheral lymphatic tissue also by means of humoral factors (39,41,57,76,111,118), although more recent studies have questioned the existence of bursal humoral factors (25,40,117).

Mammals lack a morphologically identified homologue to the bursa of Fabricius, but nevertheless express bursa-dependent types of immunity. It was therefore postulated that mammals should have a separate site for the induction of B lymphocyte differentiation, i.e. an analogue to the avian bursa of Fabricius (21,22). One such potential bursa equivalent was the gut-associated lymphatic tissues (GALT). In the rabbit these include the appendix, sacculus rotundus, Peyer's patches and possibly, the tonsils (18). Some support for this hypothesis was provided by the finding that surgical removal of the GALT combined with near-lethal whole-body irradiation in young adult rabbits resulted in a persisting impairment of antibody-mediated immunity but not in a corresponding suppression of the T lymphocytes and cell-mediated immunity (18,21). Properties of the mammalian GALT, further compatible with its hypothetical role as a bursa equivalent, include morphological similarities

between parts of the GALT and the bursa of Fabricius, especially the close relation in these lymphoepithelial tissues between lymphocytes and specialized gut epithelium (18). These similarities may, however, be coincidental and subject to other interpretations. Furthermore there are, also, suggestions that the functional mammalian bursa equivalent resides in the epithelium of the gut and skin, bone marrow, fetal liver and spleen (32,33,45,85, 91,94,95).

The GALT certainly also contains peripheral lymphatic tissue, such as parts containing T lymphocytes, and structures associated with immune functions of B lymphocytes (7,18,120,121). This can be explained by the intimate relation between the intestinal content and wall of the intestine, which should require an efficient local immune defense. Thus, material in the intestinal lumen penetrates the intestinal epithelium, especially that overlying the follicles of the GALT (7,18,121). Therefore part of the morphological appearance of the GALT may be a consequence of exposure to antigens and mitogens (121). It should be pointed out that the roles of the GALT as either central or peripheral lymphatic tissue are not mutually exclusive in our present state of knowledge.

The pioneering work of Jacobson et al. (54,55) and Süssdorf et al. (112-114) provided the first evidence for an export of cells from the GALT (Peyer's patches and appendix) to peripheral lymphatic tissues (spleen and lymph nodes) and subsequent work further supported this notion (18). However, none of these contributions directly proved the actual migration of cells from the GALT. To our knowledge the only existing direct studies are that of the present investigation reported in part previously (75) and the subsequent work of Nieuwenhuis (89). The latter demonstrated a transport of cells from the labelled appendix to the spleen and popliteal lymph nodes. Appendix labelling in that study was achieved by ^{3}H-thymidine injection either into the appendicular artery in non-irradiated rabbits or intravenously into rabbits which had previously received massive doses of X-irradiation with the appendix shielded. An emigration of cells from the GALT may be analogous to that demonstrated from the central avian lymphatic organ bursa of Fabricius (30,48,51,74). It may, however, also represent part of the extensive cell traffic between different peripheral lymphatic organs observed both in normal and immunized animals (36,46,69).

Studies of the traffic of cells between lymphatic organs have previously been performed using two principal techniques. Firstly, lymphoid cells obtained from blood, lymph or lymphatic tissue may be labelled in different

ways, e.g. by chromosome and antigenic markers or by isotopes, and then transfused into appropriate recipients (24,30,80,84,85,106). Secondly, emigration of cells from a particular tissue in situ may be studied after their local labelling by direct intra-tissue injection of an isotope such as ^{3}H-thymidine (e.g. 48,51,71,74,89).

Such local labelling techniques have the important advantage that the integrity of the labelled tissues is retained and that observed cell migrations can be expected to more closely approximate that occurring under normal conditions.

In the present investigation proliferating cells in parts of the GALT of the rabbit, i.e. the appendix, sacculus rotundus, major ileocaecal tonsil and caudal Peyer's patches, were locally labelled with ^{3}H-thymidine. A considerable emigration of lymphoid cells had previously been shown from the bursa of Fabricius in young chickens from hatching up to 6 weeks of age, but not later during the involution of this organ (51). Young, 9-week-old rabbits were therefore chosen as experimental animals because in these the peripheral lymphatic organs are undergoing rapid growth (47). Locally labelled cells from parts of the GALT representing the postulated bursa equivalent and also from parts consisting of antigen stimulated peripheral type of lymphatic tissue would especially at this time have tendency to migrate to lymphatic organs outside the locally labelled GALT, which could be explored with the specific aims presented below.

AIMS OF THE PRESENT INVESTIGATION

1. To develop a local labelling technique for the rabbit GALT and to adopt and improve techniques for the preparations of radioautographs (RAGs) of labelled lymphatic tissue sections and the staining of these. The latter was necessary because microphotometric quantitation of grain counts and cytological analysis in RAGs puts exacting demands on their quality. The main part of the different techniques arrived at are summarized in Chapter I.
2. To assess the export of ^{3}H-thymidine labelled material from the locally labelled appendix, sacculus rotundus, major ileocaecal tonsil and three caudal Peyer's patches to more orally located Peyer's patches as well as to pharyngeal tonsils, spleen, mesenteric and subcutaneous lymph nodes, thymus, femoral bone marrow, liver duodenum and ileum. For this purpose a quantitative radiochemical technique was used in which the ^{3}H content of the DNA in the target organs was assayed (Chapter II).

3. To define the morphological organization of the Peyer's patch, pharyngeal tonsil, spleen, and popliteal and mesenteric lymph nodes. Further, to establish criteria for the cytological analysis of relevant cell types in sections of these tissues. This was necessary to be able to determine accurately the localization and cytology of immigrant cells in these tissues (Chapter III).

4. To establish, whether an actual migration of cells occurs from the locally labelled GALT to the above mentioned lymphatic target organs. For this purpose a semiquantitative radioautographic technique was applied to the same material as analysed with the radiochemical method. It was then necessary to establish a minimum labelling intensity, expressed as the amount of developed silver per cell, characteristic of cells locally labelled in the GALT. Such heavily labelled cells (HLCs), which were probable immigrants from the locally labelled parts of the GALT, were characterized with respect to labelling intensity, cytology and localization in not locally labelled Peyer's patches and in pharyngeal tonsils, spleens, mesenteric and popliteal lymph nodes (Chapter IV).

CHAPTER I

METHODOLOGICAL CONSIDERATIONS

This section will summarize the experimental approach with special emphasis on the cytological and radioautographic techniques, which in many respects were considerably modified and standardized as compared to corresponding methods previously published. Details of additional techniques will be given in relavant chapters.

ANIMALS

Male albino rabbits of Swedish White Land Breed were used. They were obtained from a local breeder at 7 weeks of age, after being weaned for a week. Each animal was carefully checked for signs of infection at the arrival at the department and subsequently during the two-week adaptation period before the start of the experiment. In order to minimize stress the animals were housed in big cages (50 x 115 cm net bottoms) in groups of up to five animals with only littermates together. Meticulous care was taken to avoid draught and noise in the animal quarters and the absorbing bedding beneath the net bottom was changed daily.

The rabbits received hay and water ad libitum and twice a day a ration (about 25 ml) of the breeder's mixture of pellets (AB Harald Forss & Co., Stockholm, Sweden) and dry oats. The animals were separated until the ration had been consumed.

At the start of the experiment the rabbits, now 9-week-old and weighing 1040 $\pm$ 210 g (range 650-1490 g) were matched by weight into two groups, an experimental group of 15 animals for local labelling of the GALT and a control group of 13 animals for intraperitoneal administration of ^{3}H-thymidine. Three additional rabbits were included in the study as non-treated controls. Tissue samples from two of these were used for the control of the background in the subsequent radioautographic investigation and in the radiochemical measurements of tritium (^{3}H). The third rabbit was used for determinations of the DNA contents of the individual parts of the locally labelled GALT.

ADMINISTRATION OF ISOTOPES

1. Anatomical considerations

The caudal parts of the GALT were labelled locally with ^{3}H-thymidine in 15 rabbits. Export of radioactivity from these sites towards, inter alia, more orally localized parts of the GALT was then investigated. The anatomy of this particular part of the lymphatic system is considered below.

Only distinctly defined parts of the GALT were selected for the studies. Thus isotope was injected into the appendix, the sacculus rotundus (tonsilla iliaca), major ileocaecal tonsil and three of the Peyer´s patches. Of these sites, the sacculus rotundus was located in the wall of the ileum close to its attachment to the caecal wall. The major ileocaecal tonsil appeared as a thin extension of the sacculus rotundus located in the caecal wall (80). The Peyer´s patches were numbered in an oral direction starting from the most caudal one in the ileal wall a few centimeters oral to the ileocaecal junction. The 1st to the 3rd of these, also denominated as the "distal" Peyer´s patches, were injected locally with ^{3}H-thymidine (see below). The remaining ones in the oral direction, i.e. the 4th up to the 7th (the number of these patches varied between animals), were recovered for studies of the occurrence and localization of radioactivity transported to them from the locally labelled parts of the GALT. The two pharyngeal tonsils, easily recognized in the pharynx wall at the level of the epiglottis, were also used for the same purpose.

2. Operations

The operative technique was as follows: the animals were anaesthetized with Nembutal^{R} (Abbot, North Chicago, Ill., U.S.A.) using 20 mg/kg body weight administered intravenously and with supplementary ether inhalations. The cutaneous area between the abdominal nipples was shaved, cleaned with 0.1% Desivon^{R} solution (AB Wallco, Stockholm, Sweden) and sprayed with bacteriocidal-fungicidal Nobecutan^{R} film (AB Bofors, Mölndal, Sweden). All subsequent work was performed aseptically. A transparent sterile ethylene plastic sheet (0.02 mm thick) extensively covering the animal was attached to the sprayed area. A 5 cm midline incision beneath the major curve of the stomach was made through the sheet and the abdominal wall. Parts of the gut were successively mobilized onto the sheet. While moistened with lukewarm physiological saline, the relevant parts of the GALT (see below) were either injected into the parenchyma with ^{3}H-thymidine or sham handled but not injected. After replacement of the gut into its anatomical positions, the wound was closed with 2

layers of sutures, one closing the peritoneum and muscles, the other the skin. Finally Nobecutan^{R} was applied over the operation area. The whole procedure was finished in the individual animal in about 50 minutes.

3. Isotope injections

Tritiated thymidine (thymidine [methyl-^{3}H], spec. act. 6.7 Cimmol.; New England Nuclear Corp., Boston, Mass., U.S.A.) was diluted with 0.9% saline to 0.5 uCi/ul for local labelling of the GALT and to 0.1 uCi/ul for intraperitoneal labelling. Altogether 50 uCi ^{3}H-thymidine per kg body weight was injected with a microliter syringe fitted with a fine-gauge Kel-F Hub^{R} needle, length 50 mm, external diameter 0.25 mm (Hamilton Comp., Whittier, Cal., U.S.A.).

In the rabbits of the locally labelled group the appendix, sacculus rotundus, major ileocaecal tonsil, and the three most distally situated Peyer's patches were injected in this order with ^{3}H-thymidine. For this purpose the needle of the syringe was always inserted into, and moved through, the parenchyma parallel to the serosal layer at such a depth that its bevel was barely visible through the overlying tissue. The ^{3}H-thymidine was injected slowly and as far as possible uniformly along all the needle-paths through the tissue. At the withdrawal of the needle, cotton was lightly pressed against the serosal perforation for about10 s to minimize outflow of the isotope. The amounts of^{3}H-thymidine injected into the different organs were directly proportional to their DNA contents, as previously measured in one animal. The injection patterns were as follows: The appendix (66% of the total ^{3}H-thymidine injected) was labelled from three label tracks equally spaced around the appendiceal circumference. Each of these comprised two collinearly aligned needle-tracks which together extended along the entire length of the organ. The sacculus rotundus was labelled from 4 symmetrically spread needle-tracks radiating from a central insertion to the edge of the organ (totally 19% of the ^{3}H-thymidine), and the major ileocaecal tonsil by one central needel-track along its long axis (5% of the ^{3}H-thymidine). The three most distal Peyer's patches were each labelled by a central needle-track along their long axes (totally 10% of the ^{3}H-thymidine).

The sham-operated control animals were subjected to laparotomy and received intraperitoneally the same amount of isotope as those locally labelled. The isotope was given with a syringe so that it was evenly distributed over all superficially situated viscera, immediately before the complete closure of the abdomen. All labellings were performed between noon and 7 p.m.

DISSECTIONS

The rabbits were killed with an intravenous injection of 2 ml of Nembutal^{R} 48 hours after the labelling. The abdominal viscera were carefully inspected for signs of infection especially for coccidiotic cysts in the liver.

Since tissue samples should be collected for both radiochemical and morphological analyses the sampling technique had to be standardized in the two animal groups. Thus, among the locally labelled types of tissues, the appendix was subdivided into three regions, each about 3 cm long. From the central part of each region a slice, 5 mm wide, was taken and the slices were pooled for the radiochemical assay. For radioautography, a slice of equal width was taken from the central region only. The sacculus rotundus was hemisectioned and one half used for radiochemical assay and the other for radioautography. Of the 3 distal Peyer´s patches, the 2nd patch was divided longitudinally and one half used for the radioautographic study, while the remaining tissues were assayed radiochemically. Among the non-locally labelled types of tissues, the proximal Peyer´s patches were sampled in a similar way; one half of the 5th Peyer´s patch being used for radioautography. The left pharyngeal tonsil was included in the material of the radioautographic study and the right one used for radiochemical assay. Approximately 5 centimeters of the distal half of the ileum between the 1st and 2nd Peyer´s patch, and two equally large portions of the duodenum situated more than 5 cm caudal of the pylorus, were taken for radiochemical assay. An approximately 5 mm wide slice between them was taken for radioautography. A piece of about 1.5 g was taken from the middle lobe of the liver for radiochemical assay. From the spleen the central fourth was used for radioautography and the remaining parts for radiochemistry. Mesenteric lymph nodes adjacent to the abdominal aorta were collected and samples from these used for both types of assay, about 0.8 g being used for radiochemistry. Similarly, the popliteal lymph nodes were removed on both sides and the right together with subcutaneous axillary lymph nodes used for radiochemistry. The bone marrow of the entire right femur and about 0.7 g of the right thymic lobe were used for radiochemical studies whereas the left thymic lobe was used for histological evaluation as described in Chapter II.

The samples used for the radiochemical studies were rapidly freed from surrounding tissues, immersed in ice-cold trichloroacetic acid (5% w/v) and frozen before being further processing as described in Chapter II.

HISTOLOGICAL PREPARATION

Immediately after collection, the tissue samples for radioautography were fixed in 4% (v/v) freshly prepared calcium carbonate buffered formaldehyde solution for 48 h at room temperature. When placed in the fixative the samples from the gut were cut open, spread out and pinned on a cork plate to facilitate the subsequent orientation of the tissue in the paraffin blocks. After dehydration in graded alcohols and clearing in xylene for 30 min the tissue blocks were infiltrated with Histowax^{R} (Matheson, Coleman & Bell, East Rutherford, N.J., U.S.A.), melting point (m.p.) 50-52^{o}C, at 55^{o}C for 4 h and finally embedded in Histowax^{R} with a m.p. of 53-55^{o}C. Each sample from the GALT and the duodenum was embedded so that when cut at a microtome setting of 5 μ the sections through the intestinal parts were taken perpendicular to the plane of the flattened tissue (Fig. III:1) whereas the sections through the pharyngeal tonsils were cut in parallel with the long axis of the crypt (Fig.III :2). Four to six sections were mounted on each gelatine coated slide as further described below.

RADIOAUTOGRAPHIC TECHNIQUE

To obtain reproducible silver grain yields in the radioautographs of ^{3}H-thymidine labelled tissues, special care was taken to work under conditions of scrupulous cleanliness and uniform performance. For this purpose the microscope slides were cleaned in a washing-up machine followed by soaking for 24 hours in a bichromate sulphuric acid mixture and for 12 h in running tap water (102). After final washings in distilled water (2 x 30 min) the slides were stored in 70% ethanol until required, when they were rinsed in absolute ethanol, dried and polished with a cloth. The polished slides were coated ("subbed") with gelatine as described by Rogers (102). The above washing procedures were applied also to all other glassware used in the radioautographic procedure. To facilitate both the cleaning procedure of the slides and their following handling they were placed in glass slide racks (Wertheim Glaswerk, Wertheim, Germany) cut down to a height of 2.7 cm, in which they remained throughout all subsequent procedures except the dipping (see below).

Dewaxing and subsequent washing of the tissue sections on the slides were performed by an automatic device which submerged the slides 18 times per min into the following baths: xylene 3 x 5 min, graded (absolute, 95%,70%) ethanols 3 x 5 min each, and distilled water 5 x 3 min. While still in distilled water the slides were brought to 43^{o}C in 30 min. The water was then poured

off and the slides stored at 100% relative humidity and 43°C until the emulsion was applied.

The sections were covered with nuclear track emulsion NTB2 (Eastman Kodak, Rochester, N.Y., U.S.A.), mainly as described by Kopriwa and Le Blond (65) and Rogers (102), in a well ventilated dark room maintained at 20-22°C and 80% relative humidity. The melted emulsion was kept at 43°C and each slide individually dipped into the emulsion and then dried horizontally on a rack for 2 hours.

The slides were then replaced into the slide racks and enclosed within aluminum boxes (18.5 x 11.5 x 6.5 cm; AB Durinders Rostfria, Eskilstuna, Sweden) containing a 1 cm bottom layer of silica gel covered by a filter paper. The boxes were sealed with 5 layers of Tesa Band 651 black tape (Tesa, Beiersdorf, Germany) to prevent penetration of atmospheric water. The radioautographic slides were then exposed at 5°C for 60 days.

As in the other steps of the procedure, the processing of the radioautographs was performed as uniformly as possible. This was particularly important since the estimates of labelling intensity of the cells were based on the total amount of silver rather than the number of silver grains overlying the labelled cells (101). Before processing, the exposed radioautographs were allowed to reach room temperature overnight. They were then treated for 2 min in a D 19b developing solution (Kodak), rinsed for 10 s in distilled water, and fixed for 10 min in F24 solution (Kodak) without agitation. The degree of development was set according to Caro and van Tubergen (12) on the basis of silver grain counts performed with the same illumination (incident light) and optics as used for the subsequent microphotometric assessment of the labelling intensity of the cells. The fixed radioautographic slides were washed and agitated in distilled water for 2 min, in Hypo Clearing Agent solution (Kodak) for 2 min and finally in distilled water for 5 x 2 min. The slides were fan-dried and stored over silica gel in a refrigerator until stained. All solutions used during the processing were freshly prepared (64), filtered and then kept at 15°C ($\pm$ 0.25°C) during the whole procedure. Each bath had a volume of about 800 ml and those containing processing chemicals were renewed after 40 slides, while those consisting of only distilled water were renewed after 20 slides. Transfer time between the baths was set at 10 s. To obtain a sufficiently uniform timing of the processing in the dark room, all time intervals were indicated by means of a tape recorder.

ALCOHOLIC METHYL GREEN-PYRONIN STAINING

Staining of the tissue sections with alcoholic Methyl green-Pyronin (AMGP) was performed after the radioautographic procedure, which means that the sections had to be stained through the emulsion layer. Under these conditions current staining techniques (e.g. 35,68) had to be modified as follows to obtain a satisfactory result.

Stock solutions (2% w/v) of Methyl green (MG; E. Merck, Darmstadt, Germany; no. 42585) and Pyronin Y (PY; George T. Gurr Ltd., London, England; no. 22255) were prepared. Satisfactory stainings were obtained only when the PY dye powder appeared dark violet or dark green. Both stock solutions were allowed to "ripen" in the dark at room temperature for at least 3 days and were then filtered and extracted with chloroform (1:1). Each extraction lasted for 1 h and was repeated 4 times with the MG solution and 6 times with the PY solution. The stock solutions could then be stored for several months in a refrigerator and used again after one fresh extraction. Immediately before use, a working solution was prepared as follows: 37 ml MG, 8 ml PY, 4 ml absolute ethanol, 1 ml 100 mM sodium acetate buffer adjusted to pH 4.1 with 1 N HCl. The staining performance of this working solution gradually deteriorated when in use, but could be restored simply by adding 0.75 ml PY and 1 ml absolute ethanol every half hour.

The standard staining procedure was performed at 19-22^{o}C as follows:

1. The back of each radioautographic slide was cleaned from emulsion by rubbing with soft paper moistened with Kodak Lens Cleaner.
2. The slides were then immersed in a 70% alcoholic solution buffered with 5 mM sodium acetate with a pH of 4.1. In this and the following steps care was taken to wet each slide quickly and evenly by repeated dipping.
3. After gentle blotting, the slides were immersed in the staining solution for 5 min.
4. Excess dye solution was removed by two gentle blottings and the slides were then dried in a current of air for 5 min.
5. Rapid immersion (1 s) in 2 mM sodium acetate buffer (pH 4.1).
6. Two quick blottings (within a few seconds), fan drying (5 min) and further drying for 2 h at 37^{o}C.
7. Final dehydration as well as differentiation in n-butanol 2 x 10 min. To obtain satisfactory dehydration of the emulsion layer, the n-butanol (about 60 ml) had to be renewed for each 20th slide.
8. Immersion in frequently changed xylene 3 x 5 min and mounting in neutral embedding medium (Caedax^{R}; E. Merck).

9. The slides were dried at 43°C for 1-2 days to avoid swelling of the emulsion layer through the action of residual xylene (Dr. E. Becker, Leitz, Germany, personal communication).

If strictly adhered to, the above procedure provided a satisfactory staining result with all 8 batches of dyes so far used. Occasional failures were found to be due to either insufficiently chloroform extracted stock solutions or a too long exposure of the stained radiographs to the sodium acetate buffer (points 5 and 6). It was also confirmed that sections originating from blocks that had been infiltrated with paraffin above 56°C could not be satisfactorily stained (67).

When applied to the tissue sections covered with nuclear track emulsion the above mentioned technique gave the following staining pattern. Cell nuclei were stained with methyl green, whereas RNA containing cell structures both within the nucleus and the cytoplasm were pyroninophilic. Characteristically, cytoplasmic pyroninophilia appeared scarlet, while that of the nucleoli was definite rose. Each stain was reproducibly and distinctly localized to the respective type of cell structure which provided excellent possibilities to recognize various cell types (cf. Chapter III). This selectivity of the staining technique was retained even at widely varying staining intensities. Basal membranes remained unstained and erythrocytes, monocytes and granulocytes displayed the same pale yellow cytoplasm, as seen in unstained sections. An apparently unspecific violet staining was present only in goblet cells. The emulsion layer covering the tissue sections was only faintly rose stained provided the radioautographic slides had been treated with Hypo Clearing Agent, as described above. If this treatment was omitted there was a strong unspecific staining in the emulsion layer.

Ribonuclease test

The affinity of the Pyronin Y staining for RNA containing structures in the cells was evaluated by digesting RNA in formaldehyde fixed tissue sections from the appendix, 3rd Peyer's patch, spleen, liver and thymus, with bovine pancreatic ribonuclease (RNase; Miles-Seravac [Pty.] Ltd., Maidenhead, England; Grade III, batch 212, activity ca. 70 units/mg material, optimum activity at pH 7.7). All tissues were obtained from 9-week-old rabbits which had been injected i.v. with 0.5 uCi ^{3}H-thymidine per g body weight 1 h prior to sacrifice. The batch of RNase used was claimed by the manufacturer to be essentially protease-free. Deparaffinized 5 u sections, without emulsion, were incubated at 38°C in a continuously agitated staining jar containing

20 ml of a solution of 0.25 mg RNase/ml in redistilled water (pH adjusted to 7.4 with NaOH). To avoid inactivation of the enzyme care was taken to prepare the incubation medium in the cold and warm it to 38°C immediately before immersing the sections. One hour later the medium was replaced with a new RNase solution and the incubation allowed to continue for a further hour. After careful rinsing in redistilled water the slides were covered with nuclear track emulsion (see above), exposed for 4 weeks, processed and AMGP stained. Similarly stained radioautographs of sections treated as described above, except that RNase was omitted from the incubation medium, and of completely untreated sections were used as controls.

This procedure resulted in an almost complete removal of pyroninophilia in the RNase treated sections. A very faint staining remained in the epithelial and smooth muscle cells, whereas in the lymphoid elements virtually no staining was detectable. This was in contrast to the control-treated sections, in which the staining intensity corresponded to that of the untreated sections. The additional observation of apparently equal silver grain yields in the RNase treated as compared to the untreated and control-treated radioautographs made it further unlikely that the reduction in pyroninophilia after RNase digestion was due to action of contaminating non-specific proteases on ribonucleoproteins.

QUANTITATIVE ASSESSMENT OF THE RADIOACTIVE LABELLING INTENSITY

Heavily Labelled Cells (HLCs), as defined in Chapter IV, were sampled in radioautographs with the aid of a Leitz microscope photometer with variable measuring diaphragm (Ernst Leitz GmbH, Wetzlar, Germany) attached to a Leitz Ortholux microscope (immersion objective (P) Fl oil 105/1.32; eye-pieces Periplan GF 10 x). Stained tissue sections were selected for this purpose, using a random number table (70). Microscopic view fields were then selected at a magnification of 1225 x during a "Monte Carlo Walk" (79) across the section from a defined starting point. Both this point and the direction and distance of the "walk" between the explored view fields were determined by the use of random numbers applied to the figures on the scales and verniers of the object stage. When a view field fell partly outside a tissue section it was recorded as half a field.

Labelled cells were recognized in vertical incident-light "darkfield" illumination by means of the light reflected from the silver grains in the emulsion over the radioactive cell nucleus. The intensity of the reflected light was measured with a stabilized photomultiplier (Type MFLK, BN 5001 T,

spectral range 400-620 nm; Knott Elektronik GmbH, Munich, Germany) connected to a movingspot galvanometer with a 120 divisions scale (Type 25122; Norma GmbH, Vienna, Austria). The incident-light illumination was achieved with a 12 V 100 W halogen tungsten-filament lamp in Lamp Housing 100Z and stabilized at 5 A (Type 301-167.001 stabilizer), and a Pol-Opak illuminator with its aperture diaphragm completely opened (28) and its polarizer removed (all equipment from Leitz). The illuminator was fitted with the objective mentioned above.

During measurements the illuminating beam was passed through a Leitz 438 nm-21 nm interference filter monochromator (27) and the field diaphragm of the Pol-Opak illuminator was closed to its minimal aperture (12). During the screening for HLCs non-filtered incident light was used to illuminate the entire microscopic view field. The same illumination was also used in the subsequent closure of the diaphragms intended to mask the field surrounding the silver grains overlying the nucleus of a HLC, preparatory to the measurement of the reflected light. It was found necessary to perform this adjustment in an uniform manner which meant that the space between the margin of the measuring diaphragm and the outer margin of the silver grain accumulation over the HLC was set at 10 divisions on the scale in the observation eye-piece. Furthermore, the field diaphragm of the light source was closed down to leave an aperture with a diameter which was 20 scale divisions larger than that of the measuring diaphragm, i.e. the edges also of the diaphragms, as seen in the image plane of the objective, were separated by 10 scale divisions. Monochromatic light was employed both for the focussing, which was on the apparent top grain in the silver grain accumulation, and for the measurements of reflected light. A "blank" value, used to correct for unspecific reflection was obtained by measuring, with unchanged settings, in an adjacent emulsion area visually devoid of silver grains.

To obtain reproducible measurements the instrument was carefully calibrated by measuring the light reflection from a "standard" HLC (102) with the instrument then fitted with a fixed annular stop as field diaphragm of the light source and with another annular stop as measuring diaphragm. The aperture diameters of these were 2 mm and 3.2 mm, respectively. The photomultiplier was subsequently adjusted (approx. 950 V) to give a deflection of 76 scale divisions on the galvanometer. One scale division is denoted below as 1 arbitrary unit (AU). In order to express the amount of silver over a HLC the "blank" value was subtracted from the AU-value measured over the silver grain accumulation. Silver grain counts (in incident-light illumination) and

such microphotometric measurements of the same HLCs showed a linear relation between these two quantities up to about 50 AU. One AU, in this range, was equal to approximately 1.7 silver grains. In addition, blank values for a randomly selected sample of 5 HLCs from each of 20 radioautographic slides of the present intra-GALT labelled animal group was 1.9 ± 0.6 AU (M ± S.D.). Grain counts of non-labelled radioautographs showed a grain density of 0.34 ± 0.12 silver grains per cell. When expressed as AU this small value was disregarded in the calculations of the labelling intensities of HLCs.

There were two important potential sources of error in the measurements of reflected light from the stained radioautographs. The first of these was equivalent to what is noted as opalescense in fixed film emulsions following too rapid drying (e.g. 64). In the present system such artifacts,appeared as small depressions in the emulsion, and caused intensely light-reflecting spots, which considerably interfered with the measurements. In the present staining technique these artifacts were completely avoided by gentle drying of the AMGP stained sections in an oven before final dehydration in n-butanol.

The second source of error consisted of small water droplets ("grains") in the emulsion,preferably localized over tissue components. These "water grains" were easily confused with true silver grains in transmitted-light illumination but could be recognized in vertical illumination because they reflected this to a smaller extent than the silver grains. These "water grains", that interfered with the microphotometric measurements, were removed by carefully dehydrating the stained radioautographs in n-butanol, which by frequent changes was kept as free of water as possible.

CYTOLOGICAL CLASSIFICATION AND LOCALIZATION OF HLCs IN THE LYMPHATIC TISSUES

When the labelling intensity of a HLC had been assessed, the cell was further studied with regard to cytological type and localization in the tissue with the aid of transmitted-light bright field illumination. The condenser (condenser tops Achr. 0.90 and Apl. oil 1.25) and the objectives (oil + w 12.5/0.25 P,oil+w 25/0.65 P and (P) Fl oil 105/1.32) were used together with Periplan GF 10 x eye-pieces. When the correct localization of a HLC was to be decided within an arbitrarily subdivided tissue structure (see Chapter IV) one of the eye-pieces was fitted with a square-ruled graticule. Cytological classification was performed in Köhler type illumination with the same microscopic objective as used for the microphotometric measurements and with a total magnification of 1225 x as determined with a stage micrometer.

Important in this context was that the described development of RAGs for

microphotometric measurements in incident-light illumination resulted in smaller silver grains than required for conventional grain counts in transmitted-light (102). These smaller silver grains concealed to a lesser extent the underlying cells and thus facilitated cytological analyses. Light haloes surrounding these silver grains in the emulsion could be minimized by completely opening the aperture diaphragm. When an improved contrast of the cell image was required, the opening of the field diaphragm was reduced. The small focal depth and the high image resolution obtained through these measures were of great advantage both in minimizing the concealment by the overlying silver grains and for checking that an observed cytoplasm and a labelled nucleus belonged to the same cell. The criteria used for cytological classification of the HLCs and the histology of the examined tissues are given in Chapter III.

CHAPTER II

RADIOCHEMICAL STUDIES OF CELL MIGRATION FROM GUT-ASSOCIATED LYMPHATIC TISSUE *

As outlined in the Introduction, the suggestion (116,123) and demonstration (22) of a delineation of immunological responsiveness in birds into thymus-dependent, cell-mediated, and into antibody-mediated immunity initiated a search for a mammalian bursa equivalent. One "candidate" suggested for the bursa equivalent function is gut-associated lymphatic tissue (GALT), i.e. the appendix, sacculus rotundus, Peyer's patches and possibly tonsils, which have been extensively studied from this viewpoint in the rabbit (18,20,21,43,96,97,98,121).

In analogy with the avian bursa of Fabricius (48,51,127), these potential bursa equivalent organs may seed B lymphocytes to other lymphatic organs, especially during the development of these. At a later stage of maturity, in the immunocompetent animal, recirculation and cell traffic of both T and B lymphocytes between different organs of the lymphatic system is probably also a relevant part in the maintenance of immunological functions (36,46,69). The GALT of the rabbit may as both central (bursa equivalent) and peripheral lymphatic tissue be integrated in the traffic of cells within the lymphatic system.

The aim of this part of the study is to evaluate transport of lymphoid cell DNA from rabbit GALT after local labelling (74,75) with ^{3}H-thymidine, and to determine sites of its destination. The postulate of rabbit GALT as a bursa equivalent gives special interest to this study. However, regardless of the final solution of this controversial issue, lymphoid cell migration from GALT deserves to be studied as a general, basic event in interactions between lymphatic organs. Some of the data of our study have previously been mentioned elsewhere (75). In this chapter we will demonstrate that other lymphatic organs receive significant amounts of labelled DNA from GALT using a radiochemical method. We were, however, unable to determine whether the transport of labelled DNA occurred as cells or as DNA and/or its subunits, but it will be demonstrated in a subsequent section (Chapter IV) that at least part of this transport was in the form of ^{3}H-thymidine labelled cells.

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* This study was performed in collaboration with Dr. T.J. Linna.

MATERIAL AND METHODS

Experimental material

Details for the animal material and the design of the experiments are given in Chapter I, including the local labelling with ^{3}H-thymidine of the GALT (i.e. the appendix, sacculus rotundus, major ileocaecal tonsil and the three distal Peyer´s patches) as well as the intraperitoneal "leakage" control isotope injections.

The rabbits were sacrificed 48 hours after the labelling with ^{3}H-thymidine. The following organs were sampled: the appendix, sacculus rotundus, three distal Peyer´s patches and proximal Peyer´s patches, right pharyngel tonsil, spleen, mesenteric and subcutaneous (axillary and right popliteal) lymph nodes, thymus, femoral bone marrow, liver, duodenum and ileum between the first and second distal Peyer´s patch. In addition, samples were taken from the left thymic lobe for histological evaluation.

Extraction and radiochemical assay of DNA

Nucleic acids were extracted from the organ samples according to a modified (72) Schneider procedure (107), except for some further minor modifications. At the homogenization of the organ samples, the external cooling of the flasks in the Bühler homogenizer was accomplished with pieces of dry ice and running tap water, permitting the temperature of the homogenate to be kept close to 0^{o}C and the time of homogenization to be extended until the samples had acquired a milky appearance. This generally occurred after 3 minutes. Each organ sample in this experiment was divided into two portions after its homogenization except for the pharyngeal tonsils, proximal Peyer´s patches and subcutaneous lymph nodes, which were considered too small for measurements in duplicate. Two pieces were taken from the duodenum and each divided into portions. The duplicate samples were then carried separately through the subsequent extractions and measurements. In all steps of preparation 5% (w/v) trichloroacetic acid (TCA) was used. After centrifugation at +4^{o}C, discarding of the supernatants and washing twice, the precipitates were extracted with hot (+90^{o}C) TCA. The DNA concentrations of the extracts were measured with Burton´s (11) modification of the diphenylamine reaction (26). For the radioactivity assay, 1 ml aliquots of the TCA extracts were mixed with 10 ml of Bray´s solution (8). The measurements were performed with a Packard Tri-Carb liquid scintillation system, using equivalent samples from the two uninjected animals for background substractions. Quenching was controlled by means of the channels ratio method

(5,52). The DNA values and radioactivity measurements were used to obtain the specific activity of each organ sample, expressed as counts per minute per mg DNA, and in the case of duplicates (for the duodenum quadruplicates), given as their mean.

Calculations and their rationale

The intraperitoneal injections of ^{3}H-thymidine into the control group of rabbits were given to mimic a conceived 100% "leakage" of injected isotope from the GALT to the whole body in the locally labelled animals. This circulating ^{3}H-thymidine was assumed to distribute similarly in the animals of both experimental groups and thus to label the different tissues in similar proportions. The duodenum, which contains highly proliferative epithelium, but comparatively little organized lymphatic tissue, was considered in both animal groups being mainly labelled with "leaking" ^{3}H-thymidine. Therefore, relative activities of the different organs were calculated by dividing the specific activity of the organ with the specific activity of the duodenum of the same animal. The relative activity for the different organs, using the duodenum as the reference organ, should therefore correct for their labelling by different amounts of ^{3}H-thymidine reaching the general circulation and should be the same whether the animals had received an intraperitoneal injection or had been labelled by "leaking" isotope from the locally labelled GALT. Increased relative activity of an organ of the intra-GALT labelled as compared to the i.p. labelled group, would then indicate a transport of labelled material to this organ from the locally labelled tissue (71). To check the possibility of any accidentally increased labelling of the duodenum of the intraperitoneally labelled animals, which would affect the validity of such differences in relative activities, the relative activity was also calculated using, instead of the duodenum, the sum of the specific activity of the thymus and the femoral bone marrow as reference value.

Statistics

Means are presented together with their standard deviations in the Tables and text. The Student´s t-test was used for the evaluation of the significance of the differences between means. When necessary it was extended with the approximation of Behrens-Fisher according to Cochran (16). The paired t-test was applied to compare differences in relative activity values within the experimental group. i.e. the difference within each individual animal was calculated

and the mean difference for the whole experimental group was used for the estimation of the t value.

RESULTS

None of the animals showed any signs of peritonitis or wound infection. A few coccidiotic cysts were detected in the liver of some animals which were approximately equally distributed between the two groups. Histological examination of the thymus did not reveal signs of accidental involution in any rabbit. These findings indicate that infection and/or stress, accompanied by involution of the lymphatic system (13,29) had no major influence on our findings.

Specific activities

The locally labelled tissues (the appendix, sacculus rotundus and distal Peyer´s patches) of the locally labelled group displayed high specific activities, all significantly higher ($P < 0.001$) than the corresponding figures of the i.p. labelled control group (see Table 1). This indicates an efficient local labelling. The distribution of the specific activities among the organs showed marked differences between the two groups (Table 1). The specific activities of the proximal Peyer´s patches, pharyngeal tonsil, spleen, mesenteric lymph nodes, and subcutaneous lymph nodes were all significantly higher ($P < 0.001$; except for the spleen, $P < 0.005$) in the locally labelled group as compared to the same organs in the i.p. labelled group. All other studied organs displayed lower specific activities in the locally labelled group than in the i.p. labelled group. The significance levels of these differences were $P < 0.001$ for the duodenum, the femoral bone marrow and the thymus, $P < 0.02$ for the ileum between the first and second (distal) Peyer´s patches, but $P > 0.05$ for the liver. This suggests that more circulating ^{3}H-thymidine was generally available in the i.p. than in the locally labelled animals.

Relative activities

The mean relative activities for the different sampled organs of the locally labelled and i.p. labelled groups, using the duodenum as the reference tissue, are given in Table 2. The relative activity values of the locally labelled parts of the GALT were significantly higher ($P < 0.001$) than those of the corresponding specimens from the i.p. labelled animals. More important, the relative activities of the proximal Peyer´s patches, pharyngeal tonsil,

spleen, mesenteric lymph nodes, and subcutaneous lymph nodes of the locally labelled group were all significantly higher ($P < 0.001$) than those of the equivalent tissues in the i.p. labelled animals. This indicates a transport of labelled DNA from the locally labelled GALT to these organs. In the locally labelled group of rabbits, the mesenteric lymph nodes had a significantly higher relative activity ($P < 0.005$) than all the other lymphatic organs receiving significant quantities of labelled DNA from the locally labelled tissues. The proximal (not locally labelled) Peyer's patches had significantly higher relative activity ($P < 0.005$) than spleens, tonsils and subcutaneous lymph nodes. The differences between the relative activity for the pharyngeal tonsil, spleen and subcutaneous lymph nodes did not obtain statistical significance. The relative activities of the liver, ileum and bone marrow did not differ significantly between the locally labelled and i.p. labelled groups. The relative activity of the thymus in the i.p. labelled group was unexpectedly found to be significantly higher ($P < 0.005$) than in the locally labelled animals.

Relative activities for the various organs were also calculated, using as reference the sum of the specific activities for the thymus and the femoral bone marrow instead of the duodenum. The relative activities obtained in this way were similar to the equivalent values for the different organs when calculated using the duodenum as reference. Thus, the same significant differences were obtained with probability levels equal in value to those given in Table 2. The view of a transport of label from the GALT is thus corroborated by the results of these additional calculations.

DISCUSSION

The present study demonstrated an apparent transport of ^{3}H-labelled material from the locally ^{3}H-thymidine labelled parts of the rabbit GALT, *i.e.* the appendix, sacculus rotundus, major ileocaecal tonsil and three distal Peyer's patches to the proximal (not locally labelled) Peyer's patches, pharyngeal tonsils, spleens, and mesenteric as well as subcutaneous lymph nodes. No such transport of label was shown to the thymus, femoral bone marrow, liver and ileum. The transported label was demonstrated as ^{3}H-labelled DNA in the target organs, but the actual transport may have occurred as either cells, DNA and/or its subunits.

These conclusions were based on results of comparisons between the locally intra-GALT labelled and intraperitoneally labelled groups of animals with respect to either the mean specific or relative activity for each

type of tissue. The rationals for these comparisons have been briefly outlined in the Material and Methods section of this chapter and more extensively discussed elsewhere (71, 72). The i.p. labelled group in the present investigation was a critical control for the leakage of ^{3}H-thymidine occurring from the sites of local isotope injection in the intra-GALT labelled group, the validity of which therefore merits further consideration.

It is quite obvious that a substantial amount of such nonincorporated isotope reaches the general circulation at the local labelling of tissues (Table 1), assumably exceeding the quantity retained at the site of its application. This so-called "leakage" concerns the passage of isotope through tissue fluids into the blood capillaries and lymph vessels of the locally labelled tissue rather than a true leaking through needle-paths or small ruptures. In the present investigation the experimental model employed did not allow the magnitude of the leakage of ^{3}H-thymidine to be directly assessed. Labelling of cells, by leaking isotope, in tissues outside those locally labelled can actually largely be prevented by intravenous administration of an excess of competing non-radioactive thymidine together with interruption of the blood supply, and consequently the access of non-labelled thymidine to the locally labelled organs (e.g.125). We have, however, chosen to accept leakage of ^{3}H-thymidine at the local labelling and instead attempted to calculate its maximal impact, avoiding interference with the blood supply of rapidly proliferating lymphatic tissues and infusion of thymidine in large non-physiological amounts.

A conceived 100% ^{3}H-thymidine leakage from the locally labelled GALT was aimed at by intraperitoneal isotope administration in the control animals. The results showed that this approach was valid and actually produced an apparent overestimate of the true leakage of ^{3}H-thymidine from the locally labelled GALT. Thus, the specific activities of the rapidly proliferating duodenum and ileum were significantly higher in the i.p. labelled leakage control animals than in the locally labelled group. The possibility still existed that this difference was due to a "local labelling" of these parts of the intestine by the intraperitoneally applied isotope. The additional finding that also the extraperitoneally located thymus and femoral bone marrow showed a similar increased labelling in the i.p. labelled animals would, however, argue against this.

A transport of label was shown, using both specific and relative activities, from the locally labelled GALT to the popliteal lymph nodes, spleen and pharyngeal tonsils. These organs that are located well outside the locally labelled GALT must have received this label via the blood. However, the

mesenteric lymph nodes that showed a particularly high degree of labelling in the intra-GALT labelled group are connected to lymph vessels draining the locally labelled GALT. It can therefore not be excluded that they received this label both with the blood and lymph. The possible significance of this marked transport to the mesenteric lymph nodes is discussed further in Chapter IV.

Label locally injected in the GALT was also transported to other parts of the GALT, namely to the pharyngeal tonsils and the proximal Peyer's patches. This finding suggests that the GALT may partly function as a biological unit with cell traffic between its components. It may be argued that the high relative activity of the not locally labelled proximal Peyer's patches is due to a direct leakage of isotope from the locally labelled GALT through connecting lymph vessels. However, the anatomy of the rabbit gut is such that the smallest distance separating non-locally labelled Peyer's patches from locally labelled tissue in all cases exceeded 10 cm, rendering this possibility less likely.

Since gut-associated lymphatic tissue has been suggested to have a bursa equivalent function, it is of interest to compare traffic patterns relative to the chicken bursa of Fabricius and the mammalian GALT.

A migration to the bursa of Fabricius has so far been shown to occur in the embryo for blood borne cells (87) and for cells from the yolk sac (50), and in young animals for cells from the bone marrow (2,3). These cells are probably stem cells that differentiate to bursa lymphocytes. The bursa does not seem to be the site of "homing" for cells from other organs studied. Thus, it has not been possible to document a cell traffic from the chicken thymus to the bursa of Fabricius (49). In contrast, the Peyer's patches of the mouse (58,109,120), of the hamster (73) and of the calf (120) have clearly been shown to be homing sites for thymus-derived cells. There is even evidence for a thymus-dependence of GALT in the rabbit, the species chosen by us for study because of the work supporting GALT in this species for bursa-equivalent function (18,121). After intra-thymic labelling with ^{3}H-thymidine, and an experimental protocol similar to the one in the present study, evidence was found for a significant transport of labelled DNA from the thymus to the tonsils, also part of the GALT (72,75). Thymectomy combined with irradiation has been reported to result in a long-lasting lymphocyte depletion in a part of the rabbit appendix, sacculus rotundus and Peyer's patch corresponding to that termed "nonfollicular dense lymphatic tissue" in Chapter III (63,89,115,119). While these data indicate that part of the GALT is thymus-dependent, they do not exclude that other parts have bursa equivalent function (18,121).

It is also of interest to compare findings of this study with data on cell traffic from the bursa of Fabricius. While there are some similarities between the exports of labelled DNA from the GALT and from the bursa, such as a transport to the spleen in both cases, there is one striking difference. Thus, the lack of significant transport of labelled DNA from the GALT to the thymus in the present study contrasts to the transport of significant amounts of labelled DNA and cells from the bursa to the thymus in the chicken (4,51, 127). However, our data do not exclude an immigration of B lymphocytes into the mammalian thymus, especially since a low frequency of such cells actually has been demonstrated in this organ, e.g. in the mouse (92,100).

These differences in traffic patterns possibly indicate a functional difference in the relations of the rabbit GALT and chicken bursa to the thymus.

The radiochemical assay reflected the content of tritium incorporated in DNA in different organs, but did not reveal whether the observed transports of label to any extent occurred as cells. To further investigate this, samples of the experimental material were therefore taken for radioautographic evaluation, the results of which are presented in Chapter IV.

SUMMARY

The possible transport of cells and/or DNA and its subunits, from the gut-associated lymphatic tissue (GALT) to other organs was examined in young rabbits.

Proliferating cells in parts of the GALT, i.e. the appendix, sacculus rotundus, major ileocaecal tonsil and three distal Peyer's patches, were locally labelled with ^{3}H-thymidine. Control animals received this isotope intraperitoneally as a "leakage" control. After 48 hours, the radioactivity of extracted DNA was determined radiochemically in various organs of the two animal groups and expressed as either the specific activity (cpm per mg DNA) or as the relative activity (specific activity of an organ divided by the specific activity of the duodenum of the same animal). The locally, intra-GALT, labelled and the intraperitoneally labelled groups of animals were then compared with respect to either the mean specific or the mean relative activity for each type of tissue.

The results obtained indicated a highly significant transport of label from the locally labelled parts of the GALT to the proximal Peyer's patches and pharyngeal tonsils in the not locally labelled parts of the GALT, as well as to the spleen, mesenteric lymph nodes and subcutaneous lymph nodes. No transport of label could be demonstrated to the thymus, femoral bone marrow, liver, duodenum and ileum.

Table 1. Specific activity (c.p.m. per mg DNA) for the different organs of the intra-GALT and intraperitoneally (i.p.) labelled rabbits (Means ± S.D.).

ORGAN	ROUTE OF LABELLING		SIGNIFICANCE OF DIFFERENCE	
	intra-GALT (15)[a]	i.p. (13)[a]	t	P
Appendix	19000 ± 5400	790 ± 240	12.43	< 0.001
Sacculus rotundus	15000 ± 4600	650 ± 180	10.98	< 0.001
Distal Peyer's patches	4900 ± 2400	970 ± 170	5.93	< 0.001
Proximal Peyer's patches	1800 ± 650	940 ± 260	4.52	< 0.001
Pharyngeal tonsil	1100 ± 360	570 ± 190	4.78	< 0.001
Spleen	1200 ± 320	790 ± 180	4.13	< 0.005
Mesenteric lymph nodes	3300 ± 1400	730 ± 170	6.80	< 0.001
Subcutaneous lymph nodes (axillary + popliteal)	1100 ± 290	530 ± 150	5.86	< 0.001
Liver	840 ± 440	900 ± 370	0.40	> 0.60
Duodenum	1200 ± 270	1900 ± 480	5.25	< 0.001
Ileum	1200 ± 350	2100 ± 1200	2.98	< 0.02
Thymus	110 ± 35	240 ± 64	6.74	< 0.001
Femoral bone marrow	910 ± 280	1400 ± 270	4.24	< 0.001

[a] Number of animals.

Table 2. Relative activity[a] for the different organs of the intra-GALT and intraperitoneally (i.p.) labelled rabbits (Means ± S.D.).

ORGAN	ROUTE OF LABELLING		SIGNIFICANCE OF DIFFERENCE	
	intra-GALT (15)[b]	i.p. (13)[b]	t	P
Appendix	17.00 ± 5.50	0.44 ± 0.18	10.92	< 0.001
Sacculus rotundus	13.00 ± 4.90	0.35 ± 0.11	9.20	< 0.001
Distal Peyer's patches	4.30 ± 2.10	0.53 ± 0.17	6.37	< 0.001
Proximal Peyer's patches	1.60 ± 0.50	0.51 ± 0.15	7.06	< 0.001
Pharyngeal tonsil	1.00 ± 0.36	0.32 ± 0.15	6.25	< 0.001
Spleen	1.10 ± 0.37	0.44 ± 0.15	5.77	< 0.001
Mesenteric lymph nodes	3.00 ± 1.40	0.40 ± 0.14	6.52	< 0.001
Subcutaneous lymph nodes (axillary + popliteal)	0.92 ± 0.29	0.29 ± 0.11	7.42	< 0.001
Liver	0.78 ± 0.53	0.48 ± 0.20	1.94	> 0.05
Ileum	1.00 ± 0.26	1.20 ± 0.64	0.91	> 0.30
Thymus	0.09 ± 0.02	0.13 ± 0.04	3.13	< 0.005
Femoral bone marrow	0.80 ± 0.29	0.74 ± 0.21	0.60	> 0.50

[a] The relative activity is the specific activity (c.p.m. per mg DNA) of an organ divided by the specific activity of the duodenum (reference tissue) of the same animal. Very similar results were obtained with the thymus and femoral bone marrow combined as reference tissue (results not shown).

[b] Number of animals.

CHAPTER III

MORPHOLOGY AND CYTOLOGY OF LYMPHATIC ORGANS

The purpose of this chapter is to define the morphological organization of the lymphatic organs analysed, in radioautographs, for cells immigrating from the locally labelled parts of the GALT (cf. Chapter IV). The presentation is limited to the fifth (proximal) Peyer's patch, the pharyngeal tonsil, the spleen, as well as the popliteal and mesenteric lymph nodes.

In addition the features are described which characterize the relevant immigrant types of cells encountered as Heavily Labelled Cells (HLCs) in alcoholic methyl-green-pyronin (AMGP) stained radioautographs of sections of these organs.

The material and methods employed for this histological and cytological analysis are described in detail in Chapter I.

MORPHOLOGY OF SAMPLED TISSUES

In Figures 1-4 are given schematically the histologic organization of respectively the Peyer's patch (Fig. 1), the pharyngeal tonsil (Fig. 2), the spleen (Fig. 3) and the lymph nodes (Fig. 4). The figures summarize results of previous studies by others (see below) as well as observations on the present experimental material. In the figures the frequencies of the signs for plasma cells as well as small, medium-sized (including immature plasma cells) and large lymphocytes indicate very approximately the frequencies of these cell types in the various parts of each tissue. Detailed accounts of the histologic organization as well as reviews of the literature have previously been given for the rabbit Peyer's patch (1,62,89,99,119,121,124) pharyngeal tonsil (34), spleen, and lymph nodes (9,89,110,119). The features characterizing the various parts of each tissue are therefore only given when the definition in the present investigation departs from those in previous studies and, further, is not evident from Figures 1-4.

Peyer's patch and pharyngeal tonsil

In the 5th Peyer's patch (Fig. 1) and pharyngeal tonsil (Fig. 2) the dense lymphatic tissue in the nonfollicular region could be divided into

two parts. The larger "heterogeneous" part was characterized by a densely and irregularly arranged population of lymphoid cells which included both small and large cells. The staining of the nuclei of the small lymphocytes ranged from darkly to lightly. The smaller and peripherally situated "homogeneous" part contained a dense and regularly arranged population of small lymphocytes. The nuclei of these were more uniformly medium-darkly stained. Of particular importance in the pharyngeal tonsil, these cells could be distinguished from those in the cap of the follicular region which had more distinct nuclear outlines.

Spleen

In the spleen (Fig. 3) the "pericapillary lymphocyte sheath" was defined as the thin, relatively cell dense sheath of predominantly small and darkly stained lymphocytes surrounding the capillaries originating from the arterioles and thus continuing the "periarteriolar lymphocyte sheaths". As "perivascular lymphoreticular shell" was defined the reticular tissue immediately around the pericapillary and the terminal part of the periarteriolar lymphocyte sheaths which contained relatively densely arranged and more lightly stained small lymphocytes and immature as well as mature plasma cells.

Lymph nodes

In the lymph nodes (Fig. 4) the cortico-medullary border could be sharply delineated within one to two small lymphocyte diameters. This was possible because the nuclei of small lymphocytes in the cortex stained more darkly than in the medulla. Furthermore, the lymph vessels and sinuses were distinctly larger and more irregular in the medulla than in the cortex. A "cortical" and a "medullary junctional region" was arbitrarily defined along either side of the cortico-medullary junction. Each of these was one microscopic view field wide (i.e. approximately 122 μ) at the 1225 x magnification used. Both junctional regions were subdivided into a "proximal" and a "distal" half relative to the cortico-medullary border.

Two types of tissue could be defined in the medullary cords of the lymph nodes. The "lymphocytic tissue" contained a diversity of lymphoid cells, including such belonging to the plasma cell series. The part of the cord adjacent to the cortex sometimes consisted of "plasmacellular tissue", which was characteristically dominated by mature plasma cells, but also contained some small and medium-sized lymphoid cells.

The "centrofollicular lymphatic tissue" in the outer cortex of lymph nodes, whose medullary cords contained significant amounts of plasmacellular tissue, showed relatively dense masses of mainly small lymphocytes adjacent to the cortico-medullary border. These were termed "centrofollicular dense lymphatic tissue".

Basically, the mesenteric and popliteal lymph nodes displayed similar histologic organization, and the same nomenclature was therefore used for them. Figure 4 shows the typical morphological organization of the popliteal lymph node. Compared to the popliteal lymph nodes, the mesenteric lymph nodes had relatively more voluminous lymphatic follicles, and therefore less nonfollicular outer cortical tissue. The mid-deep cortex (i.e. paracortex) and the medullary cords also appeared proportionately better developed, while the centrofollicular dense lymphatic tissue and plasmacellular tissue were much more scarce than in the popliteal lymph nodes. The mesenteric lymph nodes in addition had a generally higher frequency of pyroninophilic lymphoid cells. All this suggests that they were more "active" than the popliteal lymph nodes.

CYTOLOGY OF SAMPLED HEAVILY LABELLED CELLS

Relevant cell types in AMGP-stained sections were defined with respect to approximate size (erythrocyte diameters, ED), nuclear structure (chromatin arrangement and nucleoli), cytoplasmic structure and pyroninophilia as well as relation between nuclear and cytoplasmic size. The descriptions of the cell types given below are in part based on information extracted from previous work in this area (e.g. 9,31,61,66,77,89,104,105,108,119,126). The following types of labelled cells in radioautographs of the various lymphatic organs were identified.

Small lymphocytes

The common type of small lymphocyte (Fig. 5a) was a roughly round, sometimes elongated cell with a diameter of 1-1.5 ED. The chromatin was aggregated into a rather coarse band along the nuclear envelope with interiorly attached clumps and into long moderately coarse trabecules fairly irregularly and openly crossing the nucleus. The trabecules exhibited angular knots of varying size with the large angular knots centrally positioned in the nuclei. The aggregated chromatin stained moderately to intensely bluish green. The chromatin structures were distinct and displayed a high staining intensity contrast to the remaining nucleus. A small to medium-sized nucleolus was

occasionally seen. The cell was either devoid of visible cytoplasm or contained a thin rim or small extensions of from moderately to heavily pyroninophilic homogeneous cytoplasm around part of the nuclear periphery.

A less common and in general slightly larger type of small lymphocyte (Fig. 5b) contained moderately intensely greenish chromatin diffusely aggregated into irregularly distributed small to moderately large "wads" and into a thin discontinuous band along the nuclear periphery. The aggregated chromatin showed a low staining intensity contrast to the greenishly tinged nuclear interspaces. The cell usually lacked cytoplasm or contained a very small amount of pyroninophilic cytoplasm.

Medium lymphocytes

The medium lymphocyte (Fig. 5c,d) was an essentially round cell with its nucleus centrally. Its diameter was 1.5-2 ED and that of the nucleus 1-1.5 ED. The chromatin was often aggregated into a thin band along the nuclear membrane and into slender trabecules which fairly regularly and densely crossed the entire nucleus. These trabecules exhibited rather small angular knots. Small chromatin clumps appeared along the peripheral chromatin band. The aggregated chromatin was distinctly delimited and stained moderately intensely green with a blue tinge. It displayed a high staining intensity contrast to the neighbouring nuclear parts (Fig. 5d). Medium lymphocytes were also found that showed the chromatin appearance of the common type of small lymphocyte (Fig. 5c). The nucleus always contained one or more small to medium-sized nucleoli. The moderately to heavily pyroninophilic cytoplasm surrounded the entire nucleus as a thin to rather broad rim. Its pyroninophilic material appeared either homogeneous or as irregular tiny knots and strings between which small unstained irregular vacuoles could occur. The cellular border appeared distinct and with a smooth outline.

Large lymphocytes

The large lymphocyte (Fig. 5e,f) was essentially rounded with a smooth to wavy outline. Its nucleus was mostly central and lightly stained. The cell diameter was 2-4 ED and the nuclear diameter 1.5-2.5 ED. The chromatin was aggregated thinly along the nuclear membrane and into very slender trabecules extending very openly and irregularly through the otherwise unstained nuclear material. Very small angular knots were found. The aggregated chromatin was distinctly delimited and moderately weakly green stained. The large lympho-

cytes could also have the chromatin appearance of medium lymphocytes. Large nucleoli occurred regularly, sometimes as broad bands, and could practically fill the cell nucleus. The appearance of the cytoplasm was principally as described for the medium lymphocyte, except that its width ranged from rather thin to broad and that it sometimes contained more abundant and even larger unstained vacuoles.

When the results of the cytologic analysis of HLCs were summarized, medium and large lymphocytes were considered together because of the difficulty to always accurately classify the transitional forms. Most HLCs in this category were, however, medium lymphocytes.

Plasma cells

The mature plasma cell (Fig. 5g) was an elongated, often egg-shaped cell, 1-1.5 ED x 1.5-2 ED, with a very darkly stained round eccentrically positioned nucleus. The nuclear diameter was 1-1.5 ED. Most of the nucleus contained aggregated chromatin as a thin to relatively coarse peripheral band and as large irregular clumps. The latter occurred both centrally as well as adherent to the peripheral chromatin band. A few, short and slender chromatin trabecules could also be seen. The aggregated chromatin was very darkly greenish blue and not clearly delineated from the other more weakly greenish blue parts of the nucleus. One small or several tiny nucleoli were frequently seen. The nucleus could also appear as in the common type of small lymphocytes, but in contrast to these cells then frequently had a nucleolus. The cytoplasm stained brilliantly pyroninophilic. It surrounded half of the nucleus as a thin rim widening at the opposite pole, where an unstained perinuclear clear region was seen. The pyroninophilic material in the cytoplasm of most cells was arranged as a regular network of fine strings with interspaced tiny vacuoles, giving the cytoplasm a finely granular appearance. The cellular outline appeared smooth and well defined.

The mature plasma cells sampled in this investigation differed from the typical immature plasma cell (Fig. 5h), a rather heterogeneous cell type. Its cytological appearance ranged from that of a medium-sized lymphocyte to near that of the mature plasma cell. Characteristically, however, the nucleus was moderately eccentered in the cell and contained one or several small to medium-sized nucleoli. The finely granular pyroninophilic cytoplasm could contain a perinuclear clear region.

Marginal zone cells

The marginal zone cell (Fig. 5i) was essentially round with a diameter of approximately 2 ED and a nuclear diameter of approximately 1.5 ED. The diffusely aggregated chromatin occurred as fairly regularly spaced, moderately large, rounded "wads" and as a thin discontinuous band along the nuclear periphery. It stained moderately intensely bluish green with a moderate contrast to the remaining greenishly tinged nuclear parts. One to several, usually medium-sized, pale nucleoli occurred. A rather thin rim of moderately pyroninophilic, homogeneous cytoplasm surrounded the nucleus more or less completely. The cellular outlines were often difficult to discern.

Reticular cells

The reticular cells (Fig. 5j) were pleomorphous, size 1-4 ED, with little or no visible cytoplasm. The shape of their usually very lightly greenishly stained nuclei were elongated with either a smooth or irregular outline. The nuclear diameters were 1-2 ED x 1.5-3 ED. Commonly, the chromatin was evenly distributed within the nucleus and appeared homogeneous or very finely granular. However, the chromatin could also be aggregated into a relatively thin band along the nuclear periphery and into a rather regular and dense network of slender trabecules, with small angular knots within the nuclear center. A few short and coarse trabecules were sometimes seem. Larger clumps of chromatin were associated with the peripheral chromatin band. The aggregated chromatin generally was well defined and stained moderately intensely bluish green. Very pale nucleoli were sometimes seen. The cytoplasm, where discernible, varied from an incomplete thin to an irregular bulky ring around the nucleus. It usually had a greyish tinge, but was occasionally slightly pyroninophilic. It could contain small dark corpuscles of stained matter or pyknotic cells.

Pyknotic cells

The pyknotic cell (Fig. 5k) was usually round with a diameter of 1-1.5 ED and lacked visible cytoplasm and nucleoli. The chromatin was diffusely aggregated into a structureless and homogeneously stained mass. This appeared either as a round corpuscle stained moderately intensely greenishly blue, or as a moderately intensely green to intensely greenishly blue ring. The chromatin ring then surrounded a large unstained region and was thinner at one pole. This chromatin often contained perpendicular fissures.

SUMMARY

The morphological organization of the fifth (proximal) Peyer's patch, pharyngeal tonsil, spleen as well as the popliteal and mesenteric lymph nodes was described.

The cytological characteristics were also given for the different cell types appearing as Heavily Labelled Cells (HLCs; cf. Chapter IV) in the AMGP-stained radioautographs of these lymphatic organs.

The established morphological and cytological criteria were utilized in the radioautographic study presented in Chapter IV. They permitted accurate cytological classification and localization of HLCs within each tissue.

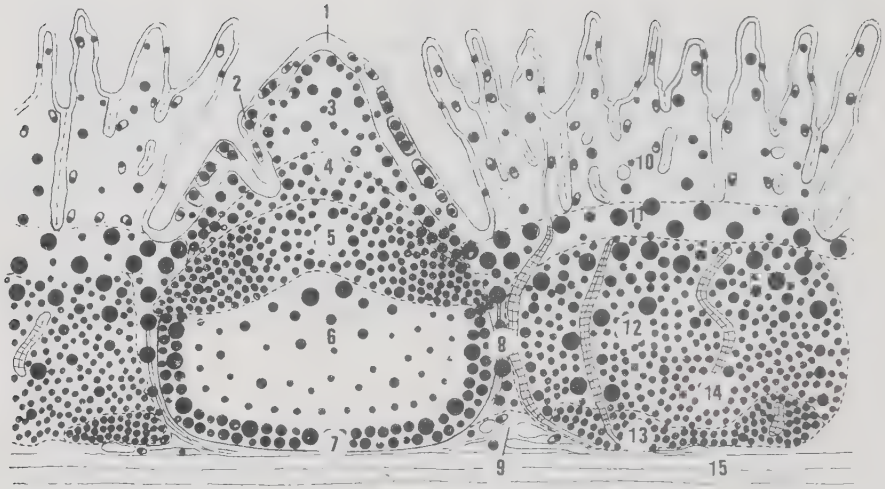

Fig. 1. Scheme of the morphologic organization of the 5th Peyer's patch. In this figure, as in Figs 2-4, is also indicated the occurrence of the different types of lymphoid cells in the various histologic structures. Small lymphocytes (●), medium-sized lymphoid cells (●), large lymphocytes (●) and plasma cells (●).

1. Lymphoepithelium
2. Lymphoepithelial alveole
3. Sublymphoepithelial tissue
4. Transitional tissue
5. Cap

(3–5: Dome shaped region)

6. Medulla
7. Cortex

(6–7: Lymphocyte lobulus)

(1–7: FOLLICULAR REGION)

8. Intermediate loose lymphatic tissue
9. Peripheral loose lymphatic tissue
10. Nonfollicular mucosa
11. Paramucosal loose lymphatic tissue
12. "Heterogeneous" part
13. "Homogeneous" part

(12–13: Nonfollicular dense lymphatic tissue)

(8–13: NONFOLLICULAR REGION)

14. Postcapillary venule
15. External muscle layers

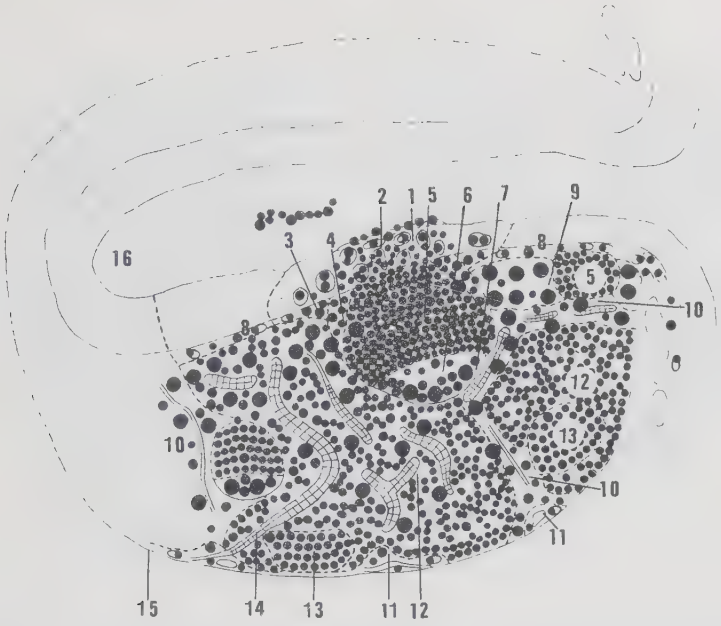

Fig. 2. Scheme of the morphologic organization of the pharyngeal tonsil. The occurrence of the lymphoid cell types is indicated as in Fig. 1.

1. Lymphoepithelium
2. Lymphoepithelial alveol
3. Sublymphoepithelial tissue
4. Transitional tissue
5. Cap
6. Medulla
7. Cortex

Lymphocyte lobulus

FOLLICULAR REGION

8. Nonfollicular mucosa
9. Paramucosal loose lymphatic tissue
10. Intermediate loose lymphatic tissue
11. Peripheral loose lymphatic tissue
12. "Heterogeneous" part
13. "Homogeneous" part

Nonfollicular dense lymphatic tissue

NONFOLLICULAR REGION

14. Postcapillary venule
15. Capsule
16. Crypt lumen

Fig. 3. Scheme of the morphologic organization of the spleen. The occurrence of the lymphoid cell types is indicated as in Fig. 1.

1. Follicular outer marginal zone
2. Follicular inner marginal zone
3. Germinal center

} FOLLICULAR REGION

4. Periarteriolar marginal zone
5. Periarteriolar lymphocyte sheath
6. Pericapillary lymphocyte sheath

} PERIARTERIOLAR REGION

} WHITE PULP

7. Perivascular lymphoreticular shell
8. Pulp cord
9. Venous sinusoid

} RED PULP

10. Arteriole (cross-hatched)
11. Arterial capillary (hatched)

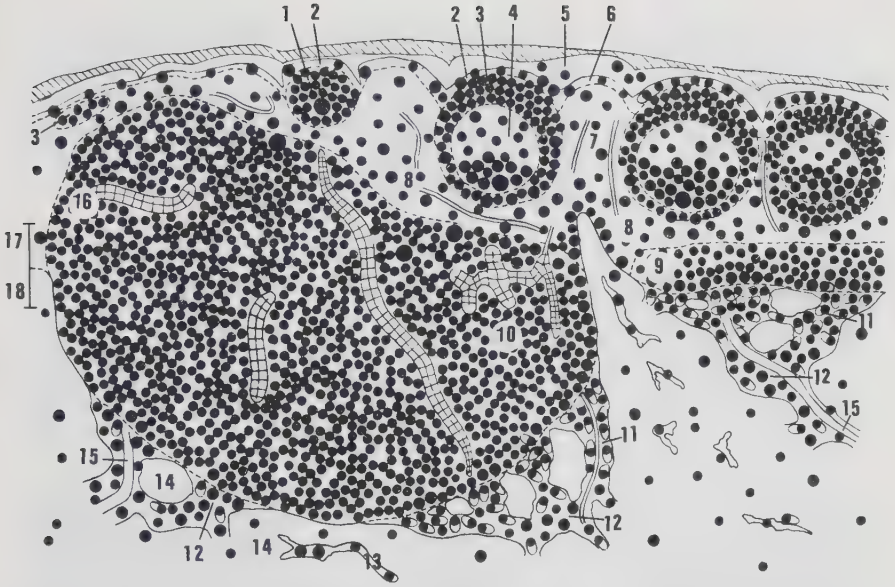

Fig. 4. Scheme of the morphologic organization of the lymph node (popliteal). The occurrence of the lymphoid cell types is indicated as in Fig. 1.

1. Lymphocyte mass
2. Follicular marginal zone
3. Corona
4. Germinal center

1:ary follicle
2:ary follicle
FOLLICULAR REGION

5. Marginal sinus
6. Interfollicular marginal zone
7. Interfollicular loose lymphatic tissue
8. Centrofollicular loose lymphatic tissue
9. Centrofollicular dense lymphatic tissue

NONFOLLICULAR REGION

OUTER CORTEX

10. MID - DEEP CORTEX (PARACORTEX)

CORTEX

11. Plasmacellular tissue of medullary cord
12. Lymphocytic tissue of medullary cord
13. Medullary reticular cell network
14. Medullary sinus

MEDULLA

15. Lymph vessel
16. Postcapillary venule
17. Approximate width of "cortical junctional region" (bar)
18. Approximate width of "medullary junctional region" (bar)

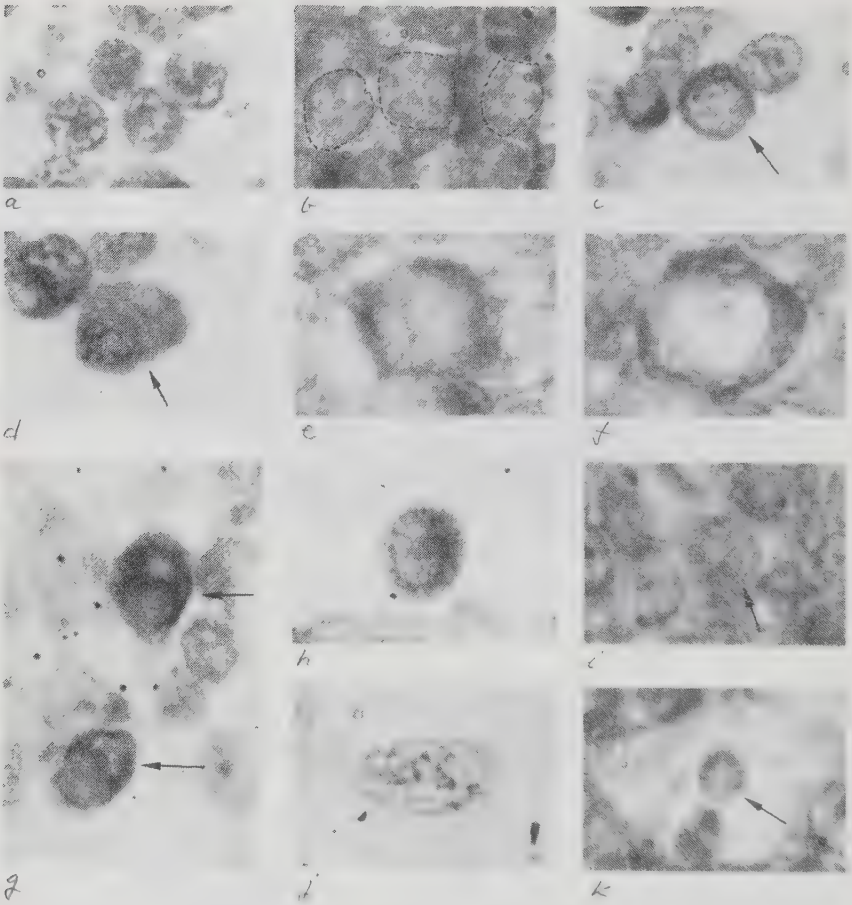

Fig. 5. Photomicrographs of cells in radioautographs considered at the cytological classifications of Heavily Labelled Cells (HLCs). Alcoholic methyl green - pyronin staining, magnification 2200 x.
a) Small lymphocytes with distinctly aggregated chromatin, b) Small lymphocytes with diffusely aggregated chromatin (encircled), c) Medium lymphocyte (arrow), d) Medium lymphocyte (arrow), thin rim of cytoplasm, e) Large lymphocyte with regularly arranged round unstained cytoplasmic vacuoles, f) Large lymphocyte with large irregular unstained cytoplasmic vacuoles, g) Mature plasma cells (arrows), h) Immature plasma cell, i) Marginal zone cell (arrow), j) Reticular cell, k) Pyknotic cell (arrow).

CHAPTER IV

RADIOAUTOGRAPHIC STUDIES OF CELL MIGRATION FROM GUT-ASSOCIATED LYMPHATIC TISSUE

Lymphatic tissues display a very complicated and unique microanatomy. It is logical that this reflects itself in functional differences. Most clearly this is seen in the separation of the lymphatic system into central and peripheral tissues (17,37,42,85). The central lymphatic tissues, i.e. the thymus, the avian bursa of Fabricius and possibly the mammalian bursa equivalent, act as microenvironments where immunocompetent lymphocytes develop from immature hemopoietic precursor cells, but they may not be directly engaged in the immune response. In contrast the peripheral lymphatic tissues contribute the microenvironment in which lymphocytes and auxilliary cell types interact with each other and with antigens. The resulting proliferation and differentiation of lymphocytes constitute the cellular basis of the immune responses. Critically important to this tissue localized immune response is the cell traffic occurring within the lymphatic system. The central lymphatic organs constantly seed immunocompetent lymphocytes to peripheral lymphatic tissues (48,51,58,72-74,125,127). Further both T and B lymphocytes migrate between different peripheral lymphatic tissues (36,46,69,74). Presence of antigen in a particular lymphatic tissue, such as the lymph nodes and the spleen, recruits circulating lymphocytes, and through the amplification process of the local immune response results in a net export of relevant effector lymphocytes to the blood and lymph (36,46,69).

In recent years much work, especially in vitro, but also using cell transfers in vivo to appropriately pretreated recipients have provided insight into the separate functions of T and B lymphocytes and macrophages as well as the role of interactions between these cells in the various types of immune responses (81-83). Little is known concerning the relations of tissue microanatomy to the traffic of cells between and within lymphatic tissue, to the interactions between antigen and lymphocytes, and to the interactions between the various cell types participating in the immune response.

Still, some information already exists relative to the preferential localization of T and B lymphocytes to, respectively, thymus-dependent and thymus-independent parts of lymphatic tissues (89,109,110,119-121). These parts,

"areas" in sections, have been associated with functions typical of either T or B lymphocytes. Thus, there is evidence strongly suggesting a certain but not absolute morphological and functional compartmentalization in the lymphatic tissues.

The lymphatic organs of the rabbit, and cell traffic between lymphatic organs in this species, are of special interest due to the fact that the rabbit appendix, sacculus rotundus and Peyer's patches have been ascribed a bursa equivalent function (18,121). Recently, a double function has been proposed for these tissues. The dome region (see Fig. 1 in Chapter III) has been proposed to have a bursa equivalent function, and the lymphocyte lobulus to be a site for B cell proliferation due to antigenic and other stimuli (121). Further, a thymus-dependent area has been proposed in the rabbit and in other species as the area corresponding largely to what has been called non-follicular dense lymphatic tissue in this chapter. The GALT must therefore clearly be regarded as at least partly peripheral lymphatic tissue.

In chapter II results were presented that indicated significant transport of label from the locally ^{3}H-thymidine labelled gut-associated lymphatic tissues (GALT), i.e. the appendix, sacculus rotundus, major ileocaecal tonsil and 3 distal Peyer's patches. This transport occurred to the not locally labelled Peyer's patches, the pharyngeal tonsils, the spleen, as well as the subcutaneous and mesenteric lymph nodes.

The quantitative radiochemical technique employed did, however, not reveal whether this transport of label actually was through live cells and, if so, the properties of these cells and their localization within the target tissue.

In the present part of the work, this problem was further studied using the same experimental material as previously described (Chapters I and II). The purpose was to evaluate the possible migration of cells, from the locally labelled parts of the GALT. For this, radioautographs of the potential target lymphatic organs for the cell traffic were prepared and a minimum labelling intensity for labelled immigrant cells (heavily labelled cells; HLCs) was defined. Such HLCs in the radioautographs were then analysed with respect to labelling intensities, frequency, cytology and localization within the lymphatic organs.

MATERIAL AND METHODS

Experimental material

The details of the animal material and the labelling with ^{3}H-thymidine have been described in Chapter I.

Those types of tissues were sampled from both animal groups to which a quantitatively significant transport of labelled DNA from the GALT was demonstrated by the radiochemical technique (Chapter II). These were the proximal 5th Peyer's patch, the left pharyngeal tonsil, the spleen, the mesenteric lymph nodes and the left popliteal lymph node. In addition locally labelled types of tissues (appendix, sacculus rotundus and 2nd Peyer's patch) as well as the duodenum were sampled but only used to examine the success of the labelling procedure.
Further details of the sampling procedure are given in Chapter I.

Histological, radioautographic and staining techniques

The histological, radioautographic and staining techniques are described in detail in Chapter I. Tissue sections (microtome setting 5 μ) were taken at 25 μ intervals and 4-6 sections were mounted on each slide. Two slides were prepared from each tissue sample in the experimental groups and 14 slides from each tissue in an unlabelled animal. They were processed for radioautography (exposure time 60 days) and then stained through the emulsion with alcoholic methyl green and pyronin (AMGP), as described in Chapter I.

Morphological and cytological analysis

The morphological organization of the relevant lymphatic organs, as well as the cytological criteria identifying different heavily labelled cell types are given in Chapter III.

Definition of Heavily Labelled Cells (HLCs)

The labelling intensity, i.e. amount of developed silver per cell, in the radioautographs (RAGs) was estimated using the quantitative microphotometrical approach as described in detail in Chapter I.

Ideally, cells labelled locally at the sites of the injection of ^{3}H-thymidine should be more heavily labelled, i.e. have more overlying silver grains in the RAGs, than cells labelled either by leaking isotope in the

intra-GALT labelled animals or the mimicked leakage of isotope in the i.p. labelled controls, due to the higher isotope concentration at the local injection site (cf. Chapter II). However, preliminary observations revealed that tissues in both the i.p. and intra-GALT labelled animals contained HLCs although these appeared considerably more numerous in the latter group.

In this situation the procedure outlined below was used to establish a practical limit in terms of labelling intensity for HLCs. The aim was to set this limit so that cells with such a minimum label or higher could, if found outside the locally labelled tissues, with a statistical approach be considered as probable emigrants from these.

Background grain counts in vertical indicent-light illumination were first determined in 8 view fields (v.f.) over each of 6 RAGs from each of the 5th Peyer's patch, pharyngeal tonsil, spleen, popliteal and mesenteric lymph nodes of the unlabelled control animal. The mean grain count per cell in a total of 6304 observed cells was 0.34 ± 0.12. Of these cells 1.5% had a count of ≥ 4 grains per cell. Therefore, cells with ≥ 4 overlying silver grains were defined as labelled cells in the RAGs of the two animal groups (6). The background silver grains were evenly distributed over the tissue sections and no evidence of positive chemography was noted.

Then 300 v.f. distributed among the RAGs of the 5th Peyer's patch of 11 animals of the i.p. labelled animal group were screened for the presence of cells with a label of ≥ 3 arbitrary photometric units (AU). The limit 3 AU was chosen for convenience and actually corresponded to approximately 5 silver grains. A total of 336 such cells were recorded. The majority of these, 97%, had a label of ≤ 12 AU, while the remaining were in the 12.5 to 85 AU range. The number of the labelled cells at successively increased AU values, when plotted in a histogram, decreased apparently asymptotically with no evidence of any peaks in the distribution.

The 5th Peyer's patch, pharyngeal tonsil, spleen, as well as the popliteal and mesenteric lymph nodes of the i.p. labelled group were then analysed for the presence of labelled cells with ≥ 12 AU, to include only cells having incorporated significant amounts of isotope. Approximately 500 v.f. were screened for each type of tissue (for details see next section). The frequency distribution of the labelled cells among these AU values was similar to that first obtained for the 5th Peyer's patch. For each tissue approximately 30% of all these labelled cells were in the ≥ 12 to < 14 AU interval, although actually a few cells with labels of up to 120 AU were recorded.

The corresponding tissues in the intra-GALT labelled group were analysed in the same way (only 60 v.f. in the mesenteric lymph nodes; see next section).

An AU limit of ≥ 14 for labelled cells was thereby chosen in an attempt to increase the possibility that locally labelled emigrants from the GALT were preferentially recorded. The histograms of the AU distributions of these labelled cells appeared similar for all tissues, with decreased cell numbers at successively higher AU values and without evidence for any peaks in the distributions. Labelling intensities up to 120 AU were recorded although the majority (90%) of the cells sampled from each tissue was in the ≥ 14 to < 50 AU interval.

To establish the minimum label in AU characteristic for locally labelled cells, the frequencies of labelled cells per v.f. at varying minimum AU values for labelled cells were calculated for the types of tissues not locally labelled. The difference in these frequencies between the intra-GALT and the i.p. labelled animals was t-tested at 0.5 AU intervals. Intra-GALT labelled animals displayed significantly more such HLCs than their i.p. labelled controls for each tissue. Maximum level of significance ($P < 0.001$; except for pharyngeal tonsil with $P < 0.005$) was achieved already for cells with ≥ 14 AU, remained unchanged up to about 30 AU and the significance levels then decreased. This statistically highly significant predominance of cells with an AU label of ≥ 14 in the intra-GALT labelled group as compared to the i.p. labelled group (Table 1) suggested that a significant fraction of these cells represented emigrant cells from the locally labelled tissues. Thus, in this investigation, a label of ≥ 14 AU appeared to give a valid statistical definition of the labelling intensity of locally labelled cells, henceforth designated Heavily Labelled Cells (HLCs).

Sampling of HLCs

View fields were positioned over the randomly selected tissue sections in a strictly randomized manner (see Chapter I) during both the establishment of minimum label for HLCs and the sampling of these in the RAGs of the intra-GALT and the i.p. labelled group.

Equal numbers of tissue sections were analysed in the two RAGs from each tissue sample of the two animal groups. In the RAGs from each animal 2 sections were studied from the 5th Peyer's patch, spleen and mesenteric lymph nodes and 4 sections from the pharyngeal tonsil and popliteal lymph node. For each type of tissue in each group approximately 500 v.f. were screened, evenly distributed among the animals. The only exception was the mesenteric lymph nodes of the locally labelled (intra-GALT) group, where only 60 v.f. were eva-

luated due to the high frequency of HLCs (cf. Table 1). In these studies each microscopic view field had an area of approximately 12 x 10^3 $/u^2$ at the 1225 x magnification used.

Presentation of results and statistics

The HLCs were classified with regard to morphologic localization and cytology (further details in Chapter III).

The labelling intensity of the HLCs in a tissue or its parts was expressed as the mean (M) ± the standard deviation (S.D.) in AU. Because cells with < 14 AU were not included in the measurements, this expression cannot be used for comparisons between mean labelling intensities of independent cell populations, e.g. to evaluate differences in labelling intensities in AU between the two animal groups. It allows, however, statistical analysis of such differences between different parts of a tissue of an experimental group, using the paired Student's t-test, where two parts of a tissue in each animal forms the pair.

For each whole tissue are given the total numbers of HLCs and cytologically specified types of HLCs as well as their frequency (number of cells per v.f.). The frequency of a cytologically specified type of HLC is also expressed as per cent of the total number of HLCs recorded in the tissue.

For each part of a tissue are given the total number of HLCs and their proportion in per cent of the total number of HLCs of either the whole tissue or of the main structure to which the part contributes.

In special cases, e.g. in the "cortical and medullary junctional regions" of the lymph nodes, the mean number of HLCs per animal in either region are presented.

The cytologically specified HLCs in a part of a tissue were given as the total number sampled and as the mean number per animal.

Differences in HLC numbers or proportions were evaluated statistically using the Student's t-test. Within the intra-GALT labelled group the paired variant of the test was used, i.e. differences were computed within each animal and the mean difference for the whole group tested against zero. Differences were accepted as significant when P values were < 0.05.

RESULTS

Frequency of HLCs in sampled tissues

Examination of RAGs from the locally labelled GALT revealed large numbers of HLCs suggesting a good labelling at the cellular level. Cells with the highest labelling intensities were usually found at the level of the upper part of the lymphocyte lobuli, i.e. in the probable vicinity of the isotope injection sites. No significant deviation from the normal histological appearance was noted in the locally labelled tissues. HLCs were rarely observed in RAGs of the duodenum of the intra-GALT or i.p. labelled groups.

Table 1 gives the frequencies of HLCs (no. HLCs per v.f.) in the not locally labelled sampled tissues of the intra-GALT labelled animal group and from the corresponding tissues of the i.p. labelled animals. The frequency figures for HLCs were always significantly higher in the intra-GALT labelled group than in the i.p. labelled group. The significance levels, also given in Table 1, were at least $P < 0.005$. This indicates a statistically significant transport of label at the cellular level to these tissues from the locally labelled part of the GALT (the appendix, sacculus rotundus, major ileocaecal tonsil and 3 distal Peyer's patches).

In the intra-GALT labelled group, HLCs were most frequent in the mesenteric lymph nodes, followed in order by the pharyngeal tonsil, 5th Peyer's patch, popliteal lymph node and spleen. When compared to the frequencies of HLCs in the spleen and in the popliteal lymph node, each of the other evaluated organs had a significantly higher frequency of HLCs, namely the 5th Peyer's patch ($P < 0.01$ resp. $P < 0.025$), pharyngeal tonsil ($P < 0.05$ resp. $P < 0.05$), and mesenteric lymph nodes ($P < 0.001$ resp. $P < 0.001$). The frequency of HLCs in the mesenteric lymph nodes was also significantly higher than in the 5th Peyer's patch ($P < 0.005$) and pharyngeal tonsil ($P < 0.02$).

HLCs in the tissues of the intraperitoneally labelled animals

The frequencies of HLCs in the different lymphatic tissues of the i.p. labelled animals (cf. Table 1) were in general too low to allow any meaningful analysis of the details of the morphological localization and labelling intensity patterns of the HLCs.

The mean label of the cells (total no. of HLCs given in parentheses) was for the 5th Peyer's patch 23 ± 13 AU (13), the pharyngeal tonsil 27 ± 11 AU (17), the spleen 18 ± 3 AU (28), the popliteal lymph node 23 ± 9 AU (22)

and the mesenteric lymph nodes 28 ± 10 AU (49).

The distribution of these HLCs among the main parts of the tissues was as follows: the follicular (31%) and nonfollicular (69%) regions of the 5th Peyer's patch, the follicular (53%) and nonfollicular (47%) regions of the pharyngeal tonsil, the white pulp (50%) and red pulp (50%) of the spleen, the cortex (91%) and medulla (9%) of the popliteal lymph node, the cortex (80%) and medulla (20%) of the mesenteric lymph nodes.

The distribution of the HLCs of the different tissues among different cytologically specified cell types are given in Tables 7-11. The majority of the HLCs were small lymphocytes.

HLCs in the 5th Peyer's patches of intra-GALT labelled animals

HLC frequency, cytology, label in AU and number of HLC contributing animals are given in Table 2 for the different structures of the 5th (i.e. not locally labelled) Peyer's patch of the intra-GALT labelled animals. Totally 391 HLCs were recorded. HLCs were found in all 15 animals. A significantly larger proportion of all HLCs sampled from the Peyer's patch appeared in its follicular region then in its nonfollicular region ($P < 0.005$).

242 (62%) of the HLCs were found in the follicular region, distributed as shown in Table 2. The majority (58%) of the HLCs occurred in the dome shaped region (the cap, transitional and sublymphoepithelial tissues), while the remaining 4% were equally distributed among the lymphoepithelium and the cortex and medulla of the lymphocyte lobulus.

149 (38%) of all recorded HLCs were located in the nonfollicular region. The details of their distribution are shown in the table. The majority of the HLCs in the nonfollicular region was found in the nonfollicular dense lymphatic tissue (20%) and the intermediate loose lymphatic tissue (9%). No HLCs were detected in the relatively voluminous "nonfollicular" epithelium. In the nonfollicular dense lymphatic tissue 79 HLCs were found in the "heterogeneous" part and 1 HLC in the "homogeneous" part.

The nonfollicular dense lymphatic tissue, in its extension perpendicular to the gut lumen and the external muscle layers beneath the serosa, was divided into thirds. Of all HLCs found in the nonfollicular dense lymphatic tissue 51 ± 30% were in the luminal, 36 ± 30% in the middle and 13 ± 18% in the serosal third (data not given in the table). The percentages HLCs in the former two (luminal and middle thirds) were significantly larger than in the serosal third ($P < 0.005$ and $P < 0.05$, respectively). However, the difference between the proportion of HLCs in the luminal and middle thirds was not signi-

ficant. Thus, the HLCs in the nonfollicular dense lymphatic tissue appeared to be more frequent in the parts luminal to the serosal third.

Table 2 also shows that the mean label of all HLCs found in the 5th Peyer's patch was 31 ± 6 AU. The mean label of HLC populations located in the different structural compartments of the organ were not significantly different from this value, nor did they differ from each other.

However, within the nonfollicular dense lymphatic tissue the mean label of the HLCs was 28 ± 12 AU (n = 13) for its luminal, 45 ± 18 AU (n = 12) for its middle and 31 ± 16 AU (n = 7) for its serosal third (data not shown in the table; n is the number of contributing animals). The HLCs of the middle third were significantly heavier labelled ($P < 0.02$) than those of the luminal third but not than those of the serosal third. The luminal third did not differ significantly from the serosal third. Thus, the HLCs of the nonfollicular dense lymphatic tissue appeared heavier labelled in at least the middle than in the luminal third.

Table 2 shows the details of the morphological localization of the cytologically specified HLCs in the 5th Peyer's patch. As demonstrated in Table 7, the majority of the HLCs appeared as small lymphocytes (88%), but heavily labelled medium-large lymphocytes (6%) and pyknotic cells (3%) were also found. In the follicular region 25 of 242 HLCs were recognized as medium-large lymphocytes. No such cells (0/149 HLCs) were identified among the HLCs in the nonfollicular region. The mean number of heavily labelled medium-large lymphocytes per animal was significantly larger ($P < 0.01$; data not given in the table) in the follicular region (1.70 ± 2.10 HLCs) than in the nonfollicular region (0 ± 0 HLCs).

Table 7 also compares the frequencies of the types of cells in the samples of HLCs from the 5th Peyer's patch in both animal groups. The frequencies of heavily labelled small lymphocytes, medium-large lymphocytes and pyknotic cells (no. cells per v.f.) were significantly higher in the intra-GALT labelled group than in the i.p. labelled animals ($P < 0.001$, $P < 0.02$ and $P < 0.01$, respectively). This indicates a significant transport of label from the locally labelled part of the GALT to the 5th Peyer's patch by cells which appeared predominantly as small lymphocytes, but also as medium-large lymphocytes and pyknotic cells, 48 hours after the local labelling.

HLCs in the pharyngeal tonsils of intra-GALT labelled animals

HLC frequency, cytology, label in AU and number of HLC contributing animals are given in Table 3 for the different structures of the pharyngeal ton-

sil of the intra-GALT labelled animals. Totally 451 HLCs were recorded. HLCs were found in all 9 animals. There was no significant difference between the proportions of HLCs sampled from the follicular and the nonfollicular region of the pharyngeal tonsil.

As shown in Table 3, 278 HLCs (62%) were found in the follicular region. The majority of these was detected in the cap and transitional tissue (54%). HLCs were found both in the cortex and medulla of the lymphocyte lobulus.

The lymphoepithelium, in its extension between the crypt lumen and the basal membrane, was divided into 3 equally sized zones, a basal, a middle and a luminal. No difference in the numbers of HLCs in these thirds was noted (5, 6 and 6 HLCs respectively; data not in the table). HLCs were localized both within the lymphoepithelial alveoli (4 HLCs) and outside these (13 HLCs).

The 173 HLCs (38%) of the nonfollicular region, as shown in Table 3, were mainly located in the nonfollicular dense lymphatic tissue (29%). Of these the "heterogeneous" part contained 86 ± 22%, a significantly larger proportion ($P < 0.005$; data not in the table) than the 14 ± 22% found in the "homogeneous" part.

In the nonfollicular region no HLCs were seen in the epithelium, which in this organ contains virtually no lymphocytes. A few HLCs were found within the crypt lumen in v.f. incidentally including its cellular material.

The mean label of all HLCs sampled from the pharyngeal tonsil was 27 ± 2 AU (Table 3). The mean label of the HLCs found in its different structural compartments did not differ significantly from that for the whole organ, nor from each other. Furthermore, the mean label 26 ± 5 AU ($n = 9$) for the "heterogeneous" part and 29 ± 9 AU ($n = 4$) for the "homogeneous" part of the nonfollicular dense lymphatic tissue did not differ significantly (data not shown in the table; n denotes the number of contributing animals).

Table 3 shows the details of the localization of the cytologically specified HLCs in the pharyngeal tonsil. As is evident from this table and the percentage figures given in Table 8 almost all (94%) of the HLCs appeared as small lymphocytes. A few medium-large lymphocytes (2%) and pyknotic cells (3%) were found.

As further shown in Table 8 the frequencies of heavily labelled small and medium-large lymphocytes (no. cells per v.f.) were significantly higher in the intra-GALT labelled than in the i.p. labelled group ($P < 0.005$ resp. $P < 0.02$). This indicates a transport of label from the locally labelled part of the GALT to the pharyngeal tonsil by cells which 48 hours after the local labelling appeared predominantly as small lymphocytes, but also as medium-large lymphocytes.

HLCs in the spleens of intra-GALT labelled animals

HLC frequency, cytology, label in AU and number of HLC contributing animals for the different structures of the spleens of the intra-GALT labelled animals are given in Table 4. A total of 234 HLCs was recorded. HLCs were found in all 15 animals. A significantly larger proportion of all HLCs sampled from the spleen appeared in its white pulp than in its red pulp ($P < 0.001$).

In the white pulp 191 HLCs (82%) were found. Of these a significantly larger proportion of HLCs ($P < 0.02$; data not in the table) was found in the follicular (65 ± 20%) than in the periarteriolar region (35 ± 20%). The HLCs of the follicular region (53%) were found in the outer marginal zone (9%), inner marginal zone (39%) and active germinal centers (4%). The HLCs of the periarteriolar region (29%) were mainly located in the periarteriolar (19%) and pericapillary (9%) lymphocyte sheaths. Thus, the majority of the HLCs in the white pulp were found in the follicular marginal zones (49%) and in the perivascular lymphocyte sheaths (28%).

The 43 HLCs of the red pulp, 18% of all spleen HLCs, were mainly found in the reticular tissue of the pulp cords (13%). As further shown in Table 4, smaller numbers of HLCs were also found in the perivascular lymphoreticular shell and in the sinusoids.

The mean label of the HLCs in the whole splenic tissue was 27 ± 4 AU (Table 4). The mean label for the white pulp and the red pulp did neither differ from this value nor from each other. However, within the white pulp, the HLCs of the periarteriolar region (34 ± 9 AU) appeared significantly heavier labelled ($P < 0.005$ at each comparison) than both the HLCs of the follicular region (25 ± 3 AU) and those of the red pulp (24 ± 6 AU).

Table 4 shows the morphological localization and Table 9 the frequencies of the cytologically specified HLCs in the spleen. The majority of these (81%) were small lymphocytes, but heavily labelled medium-large lymphocytes (2%), plasma cells (2%), marginal zone cells (5%) and pyknotic cells (4%) were also found. The mean number of heavily labelled medium-large lymphocytes per animal was significantly higher ($P < 0.02$; data not in the table) in the white pulp (0.33 ± 0.49 HLCs) than in the red pulp (0 ± 0 HLCs). Heavily labelled plasma cells were also more numerous ($P < 0.05$; data not in the table) in the white (0.27 ± 0.46 HLCs per animal) than in the red pulp (0 ± 0 HLCs). All heavily labelled marginal zone cells were located in the follicular marginal zones, none was thus found in the periarteriolar marginal zones. All pyknotic HLCs were found in the follicular region of the white pulp and in the red pulp.

Table 9 summarizes the frequencies of the cytologically specified HLCs in the spleen of both animal groups. Labelled small lymphocytes and marginal zone cells were significantly more frequent (no. cells per v.f.) in the intra-GALT than in the i.p. labelled animals ($P < 0.001$ resp. $P < 0.05$). This indicates a significant transport of label from the locally labelled part of the GALT to the spleen by cells, mostly with the appearance of small lymphocytes, but some also with marginal zone cell characteristics, 48 hours after the local labelling.

HLCs in the popliteal lymph nodes of intra-GALT labelled animals

HLC frequency, cytology, label in AU and number of HLC contributing animals for the different structures of the popliteal lymph node of the intra-GALT labelled animals are given in Table 5. A total of 245 HLCs was recorded. HLCs were found in all 14 animals. Proportionally more HLCs appeared in the cortex than in the medulla of the lymph node, but the difference was not significant. However, a significantly larger proportion of the cortical HLCs ($P < 0.001$; data not in the table) occurred in the outer cortex (80 ± 18%) than in the mid-deep cortex (20 ± 18%).

As shown in Table 5, 169 HLCs (69%) were located in the cortex, where 52% were found in the outer cortex and 17% in the mid-deep cortex.

The follicular region of the outer cortex contained 33% of all HLCs. Most of these were found in the corona (24%), the remaining HLCs were mainly located in the follicular marginal zone (3%) and the active germinal centers (5%). The thinly and the densely populated "areas" of the germinal centers contained equally many HLCs (6 each).

The nonfollicular region of the outer cortex contained 19% of all HLCs. These HLCs were rather evenly distributed among its parts. There was no significant difference between the proportions of HLCs localized in the follicular and the nonfollicular regions of the outer cortex.

The medulla contained 31% of all recorded HLCs (Table 5). These were distributed approximately equally among the plasmacellular tissue (11%) and the lymphocytic tissue (9%) of the medullary cords, and the medullary reticular cell network and sinuses (11%). All HLCs sampled from the plasmacellular tissue of the medullary cords were found within the medullary junctional region.

The results summarized in Table 5 indicate that 35% of all recorded HLCs in the popliteal lymph nodes were found in the cortico-medullary junctional region (for definition of this region, see Chapter III). Approxi-

mately equal numbers of HLCs were found in the cortical (16%) and the medullary (19%) junctional regions.

In the whole cortical junctional region (data not presented in the table), significantly more HLCs ($P < 0.02$) were sampled from its "proximal" (2.20 ± 2.40 HLCs per animal) than its "distal" half (0.57 ± 0.85 HLCs). This difference was mainly due to a significantly higher number of HLCs ($P < 0.02$) in the proximal (1.10 ± 1.20 HLCs per animal) than in the distal half (0.29 ± 0.61 HLCs) of the outer cortex part of the cortical junctional region. No such significant difference was found for the mid-deep cortex part of the cortical junctional region (1.10 ± 2.40 versus 0.29 ± 0.73 HLCs per animal). Most of the HLCs within the outer cortex part of the cortical junctional region (17/20) appeared in the centrofollicular dense lymphatic tissue.

In the medullary junctional region no significant difference was observed between the mean numbers of HLCs per animal in its proximal and its distal half. However, the part of the medullary cords located within the proximal half of the medullary junctional region contained significantly more HLCs ($P < 0.02$; data not in the table) in its plasmacellular (1.20 ± 1.50 HLCs per animal) than in its lymphocytic tissue (0 ± 0 HLCs).

Thus, significantly more HLCs were located in the proximal than in the distal half of the cortical junctional region, most notably for its part located in the outer cortex. In the proximal part of the medullary junctional region, HLCs of the medullary cords appeared preferentially in the plasmacellular tissue.

Table 5 also presents the mean label of the HLCs in the different structural compartments of the popliteal lymph node. The mean label of the HLCs of the whole organ was 28 ± 5 AU. With a few exceptions (see below) the different parts of the lymph node did neither differ from this value, nor from each other. However, closer analysis revealed a significantly lower mean label ($P < 0.005$) for the HLCs in the interfollicular loose lymphatic tissue (22 ± 6 AU) than for the neighbouring follicular region (28 ± 5 AU).

The HLCs of the cortical junctional region (34 ± 11 AU) were significantly heavier labelled ($P < 0.025$) than those of the medullary junctional region (28 ± 7 AU).

The mean label of the HLCs in the proximal half of the whole cortical junctional region (34 ± 7 AU) did not differ significantly from that of the distal half (26 ± 11 AU). However, it was significantly higher ($P < 0.05$) than that for the whole remaining cortex (27 ± 3 AU). It was also higher ($P < 0.005$) than that of the medullary HLCs (26 ± 7 AU). Most data are not given in the table.

Thus, the HLCs in the proximal half of the cortical junctional region appeared heavier labelled than those in the other parts of the popliteal lymph node.

Table 5 shows the morphological localization and Table 10 the frequency of the cytologically specified HLCs in the popliteal lymph node. The majority of the HLCs (85%) were small lymphocytes, but heavily labelled medium-large lymphocytes (5%), plasma cells (2%) and pyknotic cells (5%) were also found.

Table 10 summarizes for both animal groups the frequencies (no. cells per v.f.) of the types of cells in the sample of HLCs from the popliteal lymph node. They were significantly higher in the intra-GALT than in the i.p. labelled group for the small lymphocytes ($P < 0.001$), medium-large lymphocytes ($P < 0.025$), plasma cells ($P < 0.05$) and pyknotic cells ($P < 0.01$). This indicates a significant transport of label from the locally labelled part of the GALT to the popliteal lymph node by cells which appeared predominantly as small lymphocytes, but also as medium-large lymphocytes, plasma cells and pyknotic cells 48 hours after the local labelling.

HLCs in the mesenteric lymph nodes of intra-GALT labelled animals

HLC frequency, cytology, label in AU and number of HLC contributing animals are given in Table 6 for the different parts of the mesenteric lymph nodes of the intra-GALT labelled animals. Because only 4 v.f. were applied over the mesenteric lymph nodes of each animal, only the dominating structures of the nodes were likely to be screened for HLCs. This led to low degrees of freedom at most evaluations of the significance of the difference between the frequency or the mean label of the HLCs in the different nodular structures. None of these differences reached the level of statistical significance.

A total of 207 HLCs was recorded. HLCs were found in all 15 animals. The distribution of HLCs among the different parts of the mesenteric lymph nodes (Table 6) was similar to that found for the popliteal lymph node. Notable exceptions were within the follicular region of the mesenteric lymph nodes, where the germinal centers and the lymphocyte mass of the primary follicles contained proportionately more HLCs and within the nonfollicular region where no HLCs were found in the interfollicular loose and centrofollicular dense lymphatic tissues. Furthermore, the corticomedullary junctional region of the mesenteric lymph nodes apparently contained proportionally fewer HLCs than the same region in the popliteal lymph nodes.

There was still a tendency for HLCs to appear more frequently in the proximal than in the distal half of the cortical junctional region in the mesenteric as in the popliteal lymph nodes. Similarly, medullary cord HLCs within the medullary junctional region tended to appear preferentially in the plasmacellular tissue.

Table 6 also presents the mean label of the HLCs in the different structural compartments of the mesenteric lymph nodes. The mean label for the HLCs of the whole organ was 28 ± 6 AU. The mean labels of the HLCs in its different parts, including the cortical and medullary junctional regions, were not significantly different from that for the whole organ or from each other. However, there was a clear tendency, as in the popliteal lymph nodes, towards a heavier labelling of the HLCs in the proximal half of the cortical junctional region (37 ± 10 AU) than in the other parts of the organ.

Table 6 shows the morphological localization and Table 11 the frequency of the types of cells identified in the sample of HLCs from the mesenteric lymph nodes. The majority (69%) of the HLCs were small lymphocytes, but heavily labelled medium-large lymphocytes (8%), plasma cell (4%), reticular cells (5%) and pyknotic cells (9%) were also found. Most medium-large lymphocytes and plasma cells were found in the follicles of the outer cortex and in the medulla.

As summarized in Table 11, the frequency of the various cytologically specified HLCs (no. cells per v.f.) were all significantly larger in the intra-GALT labelled than in the i.p. labelled group, i.e. for the small lymphocytes ($P < 0.001$), medium-large lymphocytes ($P < 0.05$), plasma cells ($P < 0.02$), reticular cells ($P < 0.05$) and pyknotic cells ($P < 0.05$). These results suggest a transport of label from the locally labelled GALT to the mesenteric lymph nodes by cells which 48 hours after labelling predominantly appeared as small lymphocytes, but also as medium-large lymphocytes, plasma cells, reticular cells and pyknotic cells.

DISCUSSION

In the present investigation, part of the gut-associated lymphatic tissue (GALT) of the young rabbit, i.e. the appendix, sacculus rotundus, major ileocaecal tonsil and 3 distal Peyer's patches, was locally labelled with ^{3}H-thymidine. The local labelling technique itself and the hazards involved in this, have been discussed in detail earlier (Chapter II; ref. 71). The locally injected ^{3}H-thymidine is available in high concentration to cells proliferating in the locally labelled tissues, which results in a heavy

labelling of these cells. One problem in local labelling techniques is that ^{3}H-thymidine "leaks" into the general circulation and will label proliferating cells outside the locally labelled tissue. Using a radioautographic technique as that in the present study, it is possible to actually show that labelled cells in tissues other than those represented by the locally labelled GALT are significantly less frequent and generally less heavily labelled in the leakage controls than the cells in the same tissues in the locally labelled animals. The quantitative microphotometric "grain count" determinations in incident-light illumination permitted assessment of the labelling intensity of large numbers of cells in both animal groups. Using a statistical approach, a limit in terms of amount of developed silver per cell could be set, above which the cells with a certain probability must have been initially labelled in the locally labelled tissue, if found outside this. This approach has the advantage that this limit for HLC, i.e. a locally labelled cell, could be set so low that relatively large numbers of such cells were easily found in the various not locally labelled tissues. Furthermore, the limit for a HLC is no longer arbitrary since the effect of altering the limit could be evaluated statistically (see Material and Methods).

In the various not directly locally labelled lymphatic organs of the locally, intra-GALT, labelled animals many more HLCs, i.e. probable emigrants from the locally labelled GALT, were found than in the i.p. labelled controls 48 hours after the labelling. These differences in HLC frequencies (number of HLCs per microscopic v.f.) were all statistically highly significant. The HLCs were most frequent in the mesenteric lymph nodes followed in order by the pharyngeal tonsil, the not locally labelled 5th Peyer's patch, the popliteal lymph node and the spleen. These results then indicate that HLCs originating in the locally labelled part of the GALT migrate in significant numbers to the other assayed lymphatic organs, also to more distant sites such as the popliteal lymph nodes and the pharyngeal tonsils. They are therefore in general agreement with the data obtained with the quantitative radiochemical technique applied to the same experimental material (cf. Chapter II) and suggest that at least part of the transport of label demonstrated in that study occurred as cells.

It should be noted that the HLC frequency figures do not necessarily reflect the true frequencies of immigrants because the lymphatic organs were sampled and processed for radioautography 48 hours after the local labelling of the GALT. During this interval, many initial HLCs may have proliferated and in some of these the label may therefore have been diluted below the limit set for a HLC.

The mesenteric lymph nodes can be regarded as regional lymph nodes draining the locally labelled tissues of the GALT. The fact that the mesenteric lymph nodes showed the highest HLC frequency may be related to the possibility that they for topographical reasons could receive a disproportionate large leakage of ^{3}H-thymidine and that the i.p. labelling not adequately reflects this type of leakage. More likely, they represent an immunologically active primary target organ for the blood and lymph borne emigrants from the GALT with increased recruitment of these (18,46,69,110).

The finding that the pharyngeal tonsil and 5th Peyer's patch contained a particularly high frequency of immigrant HLCs may also be related to their high immunologically activity (7,121), further supporting the view of the GALT as a functional biological unit with cell traffic between its parts (cf. Chapter II).

It was possible, with the employed radioautographic and staining techniques, to accurately cytologically identify most HLCs, even those with densely overlying silver grains. The majority of these immigrants in all tissues examined were small lymphocytes. Small but significant numbers of heavily labelled medium-large lymphocytes, plasma cells and pyknotic cells could also be demonstrated. These findings only suggest that emigrants from the GALT appear as these cell types 48 hours after local labelling. The interval between labelling and sampling does not allow identification of the original emigrant cell types. It is likely that at least the cells labelled locally with ^{3}H-thymidine in the GALT are mainly medium-large lymphocytes, since these represent the mass of proliferating cells in lymphatic tissues (e.g. 23). Cells leaving antigen-stimulated lymph nodes are known to be predominantly large cells (46). Consequently it is also possible that the stimulation of the GALT provided by the intestinal content, including bacterial antigens (7,18,121), also in the GALT results in an emigration of especially medium-large lymphocytes. If so, their progeny develops into small lymphocytes in 48 hours or less. The possibility certainly also exists that the emigrants already when leaving the GALT are small lymphocytes. As discussed further below these emigrant cells may be both T and B lymphocytes.

Significant numbers of immigrant heavily labelled medium-large lymphocytes were detected in all examined target lymphatic organs, except the spleen. The majority of these cells were found in the follicular regions, which are generally (89,109,110,119-121) considered thymus-independent. The localization of these cells, which may partly be B lymphocytes, may have functional implications for the "plasmacellular reaction" (10,31,89,119).

The further finding of immigrant cells appearing as plasma cells in the follicular region and medulla of the lymph nodes clearly indicates a B lymphocyte migration to these thymus-independent structures from the locally labelled GALT.

Immigrants appearing as heavily labelled marginal zone cells were found in the spleens of the intra-GALT labelled animals. Contrary to previous findings (119) this cell type as defined in the present investigation was not recognized in other examined lymphatic tissues than the spleen. This discrepancy may be due to differences in cytological techniques. Whether the splenic marginal zone cells of the present study represent the same antibody forming cell precursors as previously suggested (63,89,119) remains to be demonstrated.

Also, an increased frequency of heavily labelled reticular cells were found in the mesenteric lymph nodes of the intra-GALT labelled animals, suggesting they were immigrants from the GALT. It cannot be completely excluded that they were proliferating cells labelled by possible leakage of isotope from the labelled part of the GALT as previously discussed.

Much work involving especially thymectomies and bursectomies in experimental animals and studies of human immune deficiency diseases (17,37,42,85) have defined thymus (T)-dependent and thymus-independent (B-dependent) regions in lymphatic tissues (89,109,110,119-121). In the present investigation the various lymphatic organs of intra-GALT labelled rabbits contained immigrants HLCs in both such T- and B-dependent regions. A predominance was noted for HLCs located in clearly B-dependent regions (average 61% of all HLCs in the respective organ) as compared to T-dependent regions (24%). In the particular lymphatic organs analysed the B-dependent structures, with corresponding HLC percentages in parentheses, were the follicular regions of the Peyer's patch (62%), pharyngeal tonsil (62%), and spleen (53%), as well as the follicular region and medulla of the popliteal (64%) and mesenteric (65%) lymph nodes. The T-dependent regions included the nonfollicular dense lymphatic tissues of the Peyer's patch (20%) and pharyngeal tonsil (29%), the periarteriolar region of the spleen (29%) as well as the mid-deep cortex (paracortex) of the popliteal (17%) and mesenteric lymph nodes (23%). The distribution of HLCs to B- respectively T-dependent regions are therefore remarkably similar for the different organs.

It is realized that this delineation of lymphatic tissue may not be absolute. Thus, thymus-dependence may reflect the fact that most recirculating lymphocytes are T-cells and that so-called thymus-dependent regions may be major traffic areas of such mobile lymphoid cells. However, also B lymphocyte traffic through these regions (44,46,53,69). The so-called B-dependent

regions, on the other hand, may represent areas where recirculation is not occurring to any greater extent and where mostly B lymphocytes but also some T lymphocytes may accumulate.

With these qualifications in mind, it still appears likely that both T and B lymphocytes contribute to the migrant cell population, with a probable dominance of the latter cell type. The results of this study did, however, not allow any conclusion as to whether these B lymphocytes originated from bursa equivalent tissue or from conventional peripheral lymphatic tissue in the locally labelled GALT.

The HLCs in the T-dependent regions in all examined lymphatic organs tended to have a higher labelling intensity than the HLCs in the remaining parts of these organs, significantly so in the periarteriolar region of the spleen. This could possibly be due to the fact that the T lymphocytes,constituting the majority of cells in these regions, are part of the recirculating longlived cells (36). These HLCs may also be recent immigrants in the T-dependent regions of cell traffic. In both cases the label in these HLCs is likely to have been less diluted by cell divisions. Another, but less obvious possibility is that they represent a population of particularly heavily labelled emigrants from the GALT.

In the present investigation each analysed lymphatic organ was divided into morphological compartments. Several of these only comprised a small fraction of the total tissue mass. Because the microscopic view fields analysed for the presence of HLCs were randomly positioned over the tissue sections, such small structures were rather infrequently hit and therefore only small numbers of HLCs were usually found in these. The details of the localizations and other studied features of these HLCs are mainly given in the Tables 2-6 but also in the text of the "Results" section. The findings indicate that cells migrated to many different parts of each tissue, but the small HLC samples do not usually allow further evaluations. However, a few findings were considered worthy of further comments.

Accordingly, in the lymph nodes particularly many of the immigrant HLCs were located close to the cortico-medullary border in the "junctional region", specifically in its cortical part. The immigrants in this part also appeared heavier labelled than in the rest of the lymph node. The localization of immigrants to this region may be of possible functional significance to the plasmacellular reaction (119). This region, including most of the centrofollicular dense lymphatic tissue, may also be another not yet described region of cell traffic. Its B- or T-dependence then remains to be evaluated.

As is evident from Figures 1 and 2 in Chapter III, the Peyer's patch and pharyngeal tonsil display principal morphological similarities, although the relative volumes and cytological details of many structures clearly differ. Recent work has rather thouroughly described the morphology of the Peyer's patch with emphasis on localization of T and B lymphocytes to certain of its regions and the possible functions of these regions (7,18,90,120,121). Such data do not exist for the pharyngeal tonsils. It has been suggested that at least parts of the GALT (Peyer's patches, sacculus rotundus and appendix), may contain the mammalian bursa equivalent (18,121). The findings in the present investigation of a traffic of cells from the locally labelled parts of the GALT to the not locally labelled 5th Peyer's patch and the pharyngeal tonsil therefore warrants further consideration of the localization of immigrants in these organs, with special reference to the Peyer's patch.

In the Peyer's patch two main regions have been defined. The follicular region, i.e. lymphoepithelium, sublymphoepithelial tissue, transitional tissue, cap and lymphocyte lobulus as defined in the present investigation, is mainly thymus-independent. In the nonfollicular region, the dense lymphatic tissue is thymus-dependent (90,120,121). Immigrant HLCs were found in all these structures. The structure denoted "sublymphoepithelial tissue" in this investigation apparently corresponds to the "dome" defined by Waksman et al. and proposed by these authors as a bursa-equivalent (121). The present findings of considerable numbers of immigrants in this structure in the Peyer's patch is not completely compatible with a sole bursa-equivalent function. These results instead resemble the demonstrated cell traffic from the bursa of Fabricius to the caecal tonsil of the chicken (51), an organ which may be a counterpart to a mammalian Peyer's patch (15).

The mainly T-dependent dense lymphatic tissue of the nonfollicular region in the 5th Peyer's patch showed regional differences with respect to the localization of HLCs, their labelling intensities and, as indicated in Figure 1 in Chapter III, to the occurrence of pyroninophilic lymphoid cells. The biological significance of these observations remain to be further evaluated.

The lymphocyte lobuli and the caps in the follicular region of the Peyer's patch show some morphological similarities with the germinal centers and coronas in other lymphatic organs outside the GALT. It has been suggested that lymphocyte lobuli represent a site for a more general, endotoxin stimulated, proliferation of B lymphocytes and in this way would differ from conventional germinal centers (121). In the present investigation many immigrant HLCs were found in the caps of the Peyer's patch and pharyngeal tonsils, as

well as in the coronas of the spleen and popliteal lymph nodes. Fewer, but significant, numbers of immigrant cells were found in the lymphocyte lobuli and germinal centers, except for the mesenteric lymph nodes where actually more HLCs were found in the germinal centers than in the coronas. It is possible that the coronas and caps represent a target for the immigrant HLCs, that later may be recruited to the germinal centers and lymphocyte lobuli for subsequent proliferation.

SUMMARY

The present investigation demonstrates a migration of cells from the locally labelled appendix, sacculus rotundus, major ileocaecal tonsil and the three distal Peyer's patches to the 5th Peyer's patch (not locally labelled), pharyngeal tonsil, spleen as well as mesenteric and popliteal lymph nodes. Differences and similarities were noted both within and between the sampled organs for the immigrant HLCs with respect to their localization, cytology and labelling intensity. It was not possible to evaluate all findings and only those considered most pertinent were therefore discussed. The results generally indicate that the experimental approach, with carefully selected radioautographic and cytological techniques, allows a close analysis of cell migrations between and within lymphatic tissues.

Table 1. Frequency of Heavily Labelled Cells (HLCs) in lymphatic organs of the intra-GALT and intraperitoneally (i.p.) labelled rabbits (Mean number of HLCs per view field ± S.D.).

	ORGANS AND ROUTE OF LABELLING									
	5th Peyer's patch		Pharyngeal tonsil		Spleen		Popliteal lymph node		Mesenteric lymph nodes	
	intra-GALT	i.p.	intra-GALT	i.p.	intra-Galt	i.p.	intra-GALT	i.p.	intra-GALT	i.p.
No. of animals	15	13	9[a]	9[a]	15	13	14[a]	13	15	13
No. of view fields in each group	510	507	504	504	510	507	504	507	60	507
No. of HLCs per view field	0.77 ± 0.42	0.03 ± 0.03	0.90 ± 0.60	0.03 ± 0.03	0.46 ± 0.27	0.06 ± 0.05	0.49 ± 0.29	0.04 ± 0.04	3.45 ± 2.48	0.10 ± 0.07
t	6.26		4.35		5.30		5.41		4.86	
P	< 0.001		< 0.005		< 0.001		< 0.001		< 0.001	

[a] Reduced numbers due to loss of tissue samples.

Table 2. Frequency, labelling intensity, cytology and localization of Heavily Labelled Cells (HLCs) in the proximal (5th) Peyer's patch of the intra-GALT labelled rabbits.

TISSUE STRUCTURE	HLCs				NO. OF CYTOLOGICALLY SPECIFIED HLCs[a]				
	No. of HLCs	No. of HLCs in per cent of all HLCs	No. of HLC contributing animals	Label in AU of HLCs (Mean ± S.D.)	Small lympho-cytes	Medium-large lympho-cytes	Plasma cells	Pykno-tic cells	Uniden-tified cells
Lympho-epithelium	4	1	4	24 ± 3	4				
Sublymphoepi-thelial tissue	60	15	13	28 ± 10	50	8		1	1
Transitional tissue	47	12	11	29 ± 14	39	6		1	1
Cap	119	30	15	32 ± 10	105	9		3	2
Lymphocyte lobulus	12	3	8	23 ± 7	10	2			
FOLLICULAR REGION	242	62	15	30 ± 7	208	25	0	5	4
Nonfollicular mucosa	5	1	4	33 ± 20	2		3		
Paramucosal loose lymphatic tissue	11	3	7	26 ± 10	10			1	
Intermediate loose lymphatic tissue	36	9	10	33 ± 16	36				
Peripheral loose lymphatic tissue	17	4	10	28 ± 12	15			2	
Nonfollicular dense lymphatic tissue	80	20	15	35 ± 11	75			2	3
NONFOLLICULAR REGION	149	38	15	34 ± 10	138	0	3	5	3
WHOLE ORGAN	391	100	15	31 ± 6	346	25	3	10	7

[a] No heavily labelled reticular cells were found.

Table 3. Frequency, labelling intensity, cytology and localization of Heavily Labelled Cells (HLCs) in the pharyngeal tonsil of the intra-GALT labelled rabbits.

TISSUE STRUCTURE	HLCs				NO. OF CYTOLOGICALLY SPECIFIED HLCs[a]				
	No. of HLCs	No. of HLCs in per cent of all HLCs	No. of HLC contributing animals	Label in AU of HLCs (Mean ± S.D.)	Small lympho-cytes	Medium-large lympho-cytes	Reti-cular cells	Pykno-tic cells	Uniden-tified cells
Lympho-epithelium	17	4	6	25 ± 7	14			3	
Sublymphoepi-thelial tissue	10	2	5	29 ± 14	9			1	
Transitional tissue	51	11	8	25 ± 7	48	2		1	
Cap	194	43	9	26 ± 3	185	4		2	3
Lymphocyte lobulus	6	1	4	23 ± 12	5		1		
FOLLICULAR REGION	278	62	9	26 ± 3	261	6	1	7	3
Nonfollicular mucosa	5	1	1	26 ± 7	4				1
Paramucosal loose lymphatic tissue	3	1	3	30 ± 15	3				
Intermediate loose lymphatic tissue	22	5	6	33 ± 17	22				
Peripheral loose lymphatic tissue	14	3	6	27 ± 11	14				
Nonfollicular dense lymphatic tissue	129	29	9	26 ± 4	120	2		5	2
NONFOLLICULAR REGION	173	38	9	27 ± 2	163	2	0	5	3
WHOLE ORGAN[b]	451	100	9	27 ± 2	424	8	1	12	6

[a]

[b] No heavily labelled plasma cells were found

In addition, 2 HLCs were found within the crypt lumen

Table 4. Frequency, labelling intensity, cytology and localization of Heavily Labelled Cells (HLCs) in the spleen of the intra-GALT labelled rabbits.

TISSUE STRUCTURE	HLCs				NO. OF CYTOLOGICALLY SPECIFIED HLCs						
	No. of HLCs	No. of HLCs in per cent of all HLCs	No. of HLC contribut. animals	Label in AU of HLCs (Mean ± S.D.)	Small lympho-cytes	Medium-large lympho-cytes	Plasma cells	Margi-nal zone cells	Reti-cular cells	Pykno-tic cells	Uniden-tified cells
Follicular outer marginal zone	22	9	12	26 ± 7	14	1		4		3	
Follicular inner marginal zone	92	39	15	24 ± 5	74	2	1	7		2	6
Germinal center	9	4	4	25 ± 7	7	1					1
FOLLICULAR REGION	123	53	15	25 ± 3	95	4	1	11	0	5	7
Periarteriolar marginal zone	2	1	1	24 ± 10	1						1
Periarteriolar lymphocyte sheath	45	19	11	32 ± 11	39		3				3
Pericapillary lymphocyte sheath	21	9	9	37 ± 18	20	1					
PERIARTERIOLAR REGION	68	29	14	34 ± 9	60	1	3	0	0	0	4
WHITE PULP	191	82	15	28 ± 5	155	5	4	11	0	5	11
Perivascular lympho-reticular shell	5	2	4	20 ± 4	3					2	
Reticular tissue of pulp cord	30	13	12	24 ± 8	26				1	2	1
Intralumenally in sinusoid	8	3	5	18 ± 4	6					1	1
RED PULP	43	18	12	24 ± 6	35	0	0	0	1	5	2
WHOLE ORGAN	234	100	15	27 ± 4	190	5	4	11	1	10	13

Table 5. Frequency, labelling intensity, cytology and localization of Heavily Labelled Cells (HLCs) in the popliteal lymph node of the intra-GALT labelled rabbits.

TISSUE STRUCTURE	HLCs						NO.OF CYTOLOGICALLY SPECIFIED HLCs[a,b]				
	No. of HLCs	No. of HLCs in per cent of all HLCs	No. of HLC contributing animals	No. of HLCs in cortical or medullary junctional region prox. half	dist. half	Label in AU of HLCs (Mean ± S.D.)	Small lymphocytes	Medium-large lymphocytes	Plasma cells	Pyknotic cells	Unidentified cells
Follicular marginal zone	7	3	4			36 ± 31	5	1	1		
Corona	60	24	11			27 ± 2	53	4	2	1	
Germinal center	12	5	8			27 ± 9	10	1		1	
Lymphocyte mass of 1:ary follicle	2	1	2		1	56 ± 29	2(0/1)[b]				
FOLLICULAR REGION OF OUTER CORTEX	81	33	12	0	1	28 ± 5	70(0/1)	6	3	2	0
Marginal sinus + interfollicular marginal zone	8	3	4			24 ± 7	7	1			
Interfollicular loose lymphatic tissue	13	5	7			22 ± 6	11	1		1	
Centrofollicular lymphatic tissue loose	8	3	5	1	1	31 ± 6	8(1/1)				
Centrofollicular lymphatic tissue dense	18	7	7	15	2	31 ± 9	17(14/2)	1(1/0)			
NONFOLLICULAR REGION OF OUTER CORTEX	47	19	12	16	3	28 ± 8	43(15/3)	3(1/0)	0	1	0
OUTER CORTEX	128	52	13	16	4	27 ± 4	113(15/4)	9(1/0)	3	3	0

(Continued on next page)

Table 5 (continued).

MID - DEEP CORTEX	41	17	10	15	4	32 ± 11	34(12/4)	2	0	4(3/0)	1
CORTEX	169	69	13	31	8	28 ± 5	147(27/8)	11(1/0)	3	7(3/0)	1
Plasmacellular tissue of medullary cord	27	11	8	17	10	27 ± 6	19(11/8)	1(1/0)	1(1/0)	4(2/2	2(2/0)
Lymphocytic tissue of medullary cord	22	9	8		7	24 ± 8	20(0/5)				2(0/2)
Medullary reticular cell network + sinus	27	11	9	5	7	28 ± 11	22(5/6)	1(0/1)	2	1	1
MEDULLA	76	31	13	22	24	26 ± 7	61(16/19)	2(1/1)	3(1/0)	5(2/2)	5(2/2)
WHOLE ORGAN	245	100	14	53	32	28 ± 5	208	13	6	12	6
Cortical junctional region	39	16	10	31	8	34 ± 11	35(27/8)	1(1/0)		3(3/0)	
Medullary junctional region	46	19	11	22	24	28 ± 7	35(16/19)	2(1/1)	1(1/0)	4(2/2)	4(2/2)
CORTICO-MEDULLARY JUNCTIONAL REGION	85	35	13	53	32	30 ± 7	70(43/27)	3(2/1)	1(1/0)	7(5/2)	4(2/2)

[a]

[b] No heavily labelled reticular cells were found.

Number of HLCs in proximal versus distal halves of cortical or medullary junctional region are given in parenthesis.

Table 6. Frequency, labelling intensity, cytology and localization of Heavily Labelled Cells (HLCs) in the mesenteric lymph nodes of the intra-GALT rabbits.

TISSUE STRUCTURE	HLCs						NO. OF CYTOLOGICALLY SPECIFIED HLCs[a,b]				
	No. of HLCs	No. of HLCs in per cent of all HLCs	No. of HLC contributing animals	No. of HLCs in cortical or medullary junctional region prox. half	dist. half	Label in AU of HLCs (Mean ± S.D.)	Small lymphocytes	Medium-large lymphocytes	Plasma cells	Reticular cells	Pyknotic cells
Follicular marginal zone	10	5	2			27 ± 17	6				
Corona	18	9	5			23 ± 8	14	1	1		2
Germinal center	30	14	5			29 ± 12	21	5			4
Lymphocyte mass of l:ary follicle	15	7	4			25 ± 5	9	2	2	2	
FOLLICULAR REGION OF OUTER CORTEX	73	35	8	0	0	27 ± 4	50	8	3	2	6
Marginal sinus + interfollicular marginal zone	16	8	2			23 ± 7	10		1	4	1
Interfollicular loose lymphatic tissue	0										
Centrofollicular lymphatic tissue loose	7	3	2	6		28 ± 0	6(5/0)[b]			1(1/0)	
Centrofollicular lymphatic tissue dense	0										
NONFOLLICULAR REGION OF OUTER CORTEX	23	11	4	6	0	26 ± 5	16(5/0)	0	1	5(1/0)	1
OUTER CORTEX	96	46	9	6	0	28 ± 4	66(5/0)	8	4	7(1/0)	7

(Continued on next page)

Table 6 (continued).

MID - DEEP CORTEX	48	23	11	5	0	32 ± 12	33(5/0)	1	0	2	8
CORTEX [a]	145	70	14	11	0	30 ± 7	100(10/0)	9	4	9(1/0)	15
Plasmacellular tissue of medullary cord	14	7	4	4	10	23 ± 4	10(3/7)	1(1/0)	1(0/1)		2(0/2)
Lymphocytic tissue of medullary cord	26	13	8	5		25 ± 7	17(4/0)	4(1/0)	2	2	1
Medullary reticular cell network + sinus	22	11	5		2	25 ± 8	16(0/1)	3(0/1)	1		
MEDULLA	62	30	11	9	12	25 ± 6	43(7/8)	8(2/1)	4(0/1)	2	3(0/2)
WHOLE ORGAN	207	100	15	20	12	28 ± 6	143	17	8	11	18
Cortical junctional region	11	5	3	11		37 ± 10	10(10/0)			1(1/0)	
Medullary junctional region	21	10	6	9	12	21 ± 4	15(7/8)	3(2/1)	1(0/1)		2(0/2)
CORTICO-MEDULLARY JUNCTIONAL REGION	32	15	8	20	12	26 ± 11	25(17/8)	3(2/1)	1(0/1)	1(1/0)	2(0/2)

[a]

[b] Ten HLCs with unidentified cytology are not given in the table. One HLC in cortex was found in an unidentitiable structure.

Number of HLCs in proximal versus distal halves of cortical or medullary junctional region are given in parenthesis.

Table 7. Frequencies of cytologically specified types of HLCs (CST-HLCs) in the proxim (5th) Peyer's patch of the intra-GALT and intraperitoneally (i.p.) labelled rab

CYTOLOGICALLY SPECIFIED TYPE OF HLCs	INTRA-GALT LABELLED ANIMALS n = 15[a] No. of CST-HLCs in per cent of all HLCs	INTRA-GALT LABELLED ANIMALS n = 15[a] No. of CST-HLCs per view field (M ± S.D.)	I.P. LABELLED ANIMALS n = 13 No. of CST-HLCs in per cent of all HLCs	I.P. LABELLED ANIMALS n = 13 No. of CST-HLCs per view field (M ± S.D.)	NO. OF CST-HLCs PER VIEW FIELD (intra-GALT versus i.p.) P
Small lymphocytes	88	0.616 ± 0.399	77	0.020 ± 0.026	< 0.001
Medium-large lymphocytes	6	0.049 ± 0.062	8	0.002 ± 0.007	< 0.02
Plasma cells	1	0.006 ± 0.016	0	0 ± 0	> 0.05
Reticular cells	0	0 ± 0	8	0.002 ± 0.007	> 0.05
Pyknotic cells	3	0.020 ± 0.021	0	0 ± 0	< 0.01
Unidentified cells	2	———	8	———	———

[a] Number of animals

Table 8. Frequencies of cytologically specified types of HLCs (CST-HLCs) in the pharyng tonsil of the intra-GALT and intraperitoneally (i.p.) labelled rabbits.

CYTOLOGICALLY SPECIFIED TYPE OF HLCs	INTRA-GALT LABELLED ANIMALS n = 9[a] No. of CST-HLCs in per cent of all HLCs	INTRA-GALT LABELLED ANIMALS n = 9[a] No. of CST-HLCs per view field (M ± S.D.)	I.P. LABELLED ANIMALS n = 9 No. of CST-HLCs in per cent of all HLCs	I.P. LABELLED ANIMALS n = 9 No. of CST-HLCs per view field (M ± S.D.)	NO. OF CST-HLCs PER VIEW FIELD (intra GALT versus i.p.) P
Small lymphocytes	94	0.841 ± 0.546	100	0.034 ± 0.029	< 0.005
Medium-large lymphocytes	2	0.016 ± 0.017	0	0 ± 0	< 0.02
Plasma cells	0	0 ± 0	0	0 ± 0	> 0.05
Reticular cells	0.2	0.002 ± 0.005	0	0 ± 0	> 0.05
Pyknotic cells	3	0.024 ± 0.040	0	0 ± 0	> 0.05
Unidentified cells	1	———	0	———	———

[a] Number of animals.

Table 9. Frequencies of cytologically specified types of HLCs (CST-HLCs) in the spleen of the intra-GALT and intraperitoneally (i.p.) labelled rabbits.

CYTOLOGICALLY SPECIFIED TYPE OF HLCs	INTRA-GALT LABELLED ANIMALS n = 15[a] No. of CST-HLCs in per cent of all HLCs	INTRA-GALT LABELLED ANIMALS n = 15[a] No. of CST-HLCs per view field (M ± S.D.)	I.P. LABELLED ANIMALS n = 13 No. of CST-HLCs in per cent of all HLCs	I.P. LABELLED ANIMALS n = 13 No. of CST-HLCs per view field (M ± S.D.)	NO. OF CST-HLCs PER VIEW FIELD (intra-GALT versus i.p.) P
Small lymphocytes	81	0.329 ± 0.236	75	0.041 ± 0.037	< 0.001
Medium-large lymphocytes	2	0.010 ± 0.014	11	0.006 ± 0.015	> 0.05
Plasma cells	2	0.008 ± 0.013	0	0 ± 0	> 0.05
Marginal zone cells	5	0.022 ± 0.028	4	0.002 ± 0.007	< 0.05
Reticular cells	0.4	0.002 ± 0.008	4	0.002 ± 0.007	> 0.05
Pyknotic cells	4	0.020 ± 0.029	7	0.004 ± 0.010	> 0.05
Unidentified cells	6	———	0	———	——

[a] Number of animals.

Table 10. Frequencies of cytologically specified types of HLCs (CST-HLCs) in the popliteal lymph node of the intra-GALT and intraperitoneally (i.p.) labelled rabbits.

CYTOLOGICALLY SPECIFIED TYPE OF HLCs	INTRA-GALT LABELLED ANIMALS n = 14[a] No. of CST-HLCs in per cent of all HLCs	INTRA-GALT LABELLED ANIMALS n = 14[a] No. of CST-HLCs per view field (M ± S.D.)	I.P. LABELLED ANIMALS n = 13 No. of CST-HLCs in per cent of all HLCs	I.P. LABELLED ANIMALS n = 13 No. of CST-HLCs per view field (M ± S.D.)	NO. OF CST-HLCs PER VIEW FIELD (intra-GALT versus i.p.) P
Small lymphocytes	85	0.413 ± 0.268	91	0.036 ± 0.037	< 0.001
Medium-large lymphocytes	5	0.026 ± 0.032	5	0.002 ± 0.007	< 0.025
Plasma cells	2	0.012 ± 0.018	0	0 ± 0	< 0.05
Reticular cells	0	0 ± 0	0	0 ± 0	> 0.05
Pyknotic cells	5	0.024 ± 0.024	5	0.002 ± 0.007	< 0.01
Unidentified cells	2	———	0	———	——

[a] Number of animals.

Table 11. Frequencies of cytologically specified types of HLCs (CST-HLCs) in the mesent lymph nodes of the intra-GALT and intraperitoneally (i.p.) labelled rabbits.

CYTOLOGICALLY SPECIFIED TYPE OF HLCs	INTRA-GALT LABELLED ANIMALS n = 15[a]		I.P. LABELLED ANIMALS n = 13		NO. OF CST-HLCs PER VIEW FIELD (intra-GALT versus i.p.) P
	No. of CST-HLCs in per cent of all HLCs	No. of CST-HLCs per view field (M ± S.D.)	No. of CST-HLCs in per cent of all HLCs	No. of CST-HLCs per view field (M ± S.D.)	
Small lymphocytes	69	2.383 ± 1.500	71	0.069 ± 0.058	< 0.001
Medium-large lymphocytes	8	0.283 ± 0.452	6	0.006 ± 0.011	< 0.05
Plasma cells	4	0.133 ± 0.160	4	0.004 ± 0.010	< 0.02
Reticular cells	5	0.183 ± 0.275	12	0.012 ± 0.020	< 0.05
Pyknotic cells	9	0.300 ± 0.425	6	0.006 ± 0.011	< 0.05
Unidentified cells	5	———	0	———	———

[a] Number of animals.

GENERAL SUMMARY

The main purpose of the present investigation was to establish to what extent there is a migration of cells from the gut-associated lymphatic tissue (GALT), i.e. the appendix, sacculus rotundus, major ileocaecal tonsil and Peyer's patches to other, especially lymphatic, organs in the rabbit. Attention was also directed to characteristics of possible migrant cells, including localization within target organs and cytology.

CHAPTER I. METHODOLOGICAL CONSIDERATIONS

Chapter I was devoted to a general consideration of the experimental material and methods.

I:1. Animals and local labelling technique

Young, 9-week-old rabbits received injections of ^{3}H-thymidine into the appendix, sacculus rotundus, major ileocaecal tonsil and caudal Peyer's patches (intra-GALT) to locally label proliferating cells. Control animals received ^{3}H-thymidine intraperitoneally (i.p.) to mimic leakage of isotope at the local labelling. Special care was directed to perfect as far as possible the labelling technique as well as the pre- and postoperative handling of the rabbits. This was of particular importance in view of the well established sensitivity of the lymphatic system to stress situations.

I:2. Sampling of tissues and radioautographic technique

The sampling of tissues 48 h after the labellings for subsequent measurement of ^{3}H-thymidine incorporation into DNA (the radiochemical technique) and for the radioautographic procedure was strictly standardized. The radiochemical technique is dealt with in Chapter II. The radioautographic technique was based on a conventional liquid emulsion "dipping" technique. However, the technique had to be modified in order to minimize artifacts and permit reproducible grain count determinations. This was important because a semiquantitative approach was utilized where the amount of developed silver over isotopically labelled cells was measured photometrically as the light reflected in incident-light illumination. The instrumentation itself and the practical details of its use were described. One advantage with

the microphotometric technique was the rapid and accurate determinations of developed silver over labelled cells in the sections. Another advantage, inherent in the technique, was the possibility to evaluate the cytology of even intensely labelled cells in the radioautographs because the size of the overlying silver grains could be kept small and thus less concealing.

I:3. Methyl green and Pyronin staining

To facilitate further the cytological analyses of radioautographs of lymphatic sections, a procedure was elaborated for alcoholic methyl green and pyronin staining of tissue sections through the processed radioautographic emulsions. The staining technique, in which the pyronin component was highly selective for cell structures containing RNA, gave excellent results and permitted the cytological classification of ^{3}H-thymidine labelled cells (see below).

CHAPTER II. RADIOCHEMICAL STUDIES OF CELL MIGRATION FROM GUT-ASSOCIATED LYMPHATIC TISSUE

The specific activities of DNA (counts per min per mg DNA) extracted from the various organs and tissues of intra-GALT or i.p. labelled rabbits 48 h after labelling were determined. Comparisons of the specific activities of the various organs showed that these were higher for the non-lymphatic tissues (liver, duodenum, ileum) and the thymus and femoral bone marrow of the i.p. labelled animals than for corresponding tissues in the intra-GALT labelled animals. This indicated that the isotope leakage control provided by intraperitoneal ^{3}H-thymidine administration reasonably well mimicked the actual leakage at the local (intra-GALT) labelling and if anything over-estimated it.

All other not locally labelled lymphatic organs, i.e. the proximal (orally located) Peyer's patches, pharyngeal tonsils, spleens, subcutaneous (axillary and popliteal) lymph nodes and mesenteric lymph nodes, showed significantly higher specific activities in the intra-GALT than in the i.p. labelled animal group. The relative activities, i.e. the quotients between the specific activities of the respective organ and that of the reference tissue duodenum were similarly higher for these organs.

These results suggest a significant transport of the ^{3}H-label as either whole cells, DNA or its subunits from the locally labelled GALT to the mesenteric lymph nodes, not locally labelled Peyer's patches, spleen, pharyngeal tonsil and subcutaneous (axillary and popliteal) lymph nodes. No such transport was demonstrated to the thymus or femoral bone marrow.

CHAPTER III. MORPHOLOGY AND CYTOLOGY OF LYMPHATIC ORGANS

On the basis of the present experimental material together with data published by others, schemes were presented for the morphologic organization of the rabbit Peyer's patch, the pharyngeal tonsil, the spleen, and the lymph nodes (popliteal and mesenteric) into several well defined substructures. A number of these definitions depart from those given previously.

In addition criteria were given for the classification of relevant cell types in these lymphatic organs, i.e. those cells which were recognized as heavily labelled cells in alcoholic methyl green and pyronin stained radioautographs. Characteristic cytological features allowed the accurate distinction of small, medium-sized and large lymphocytes, immature and mature plasma cells, marginal zone cells (in the spleen only), reticular cells and pyknotic cells.

These definitions of morphology and cytology of the respective lymphatic organs served as a prerequisite for the subsequent study of cell migration with the radioautographic approach (see below).

CHAPTER IV. RADIOAUTOGRAPHIC STUDIES OF CELL MIGRATION FROM GUT-ASSOCIATED LYMPHATIC TISSUE

Chapter IV represents an inquiry into the possibility that the transport of label demonstrated with the radiochemical approach (Chapter II) from the locally labelled parts of the GALT to the proximal 5th Peyer's patch, the pharyngeal tonsil, the spleen, the mesenteric lymph nodes and popliteal lymph nodes occurred at least partly as the migration of whole cells. The immigrants into these lymphatic organs were studied with respect to frequency, localization within each tissue, cell type and labelling intensity.

IV:1. Definition of minimum labelling intensity of migrant cells

Cells locally labelled in the GALT should ideally become more intensively labelled, than cells in the remaining parts of the lymphatic system labelled by ^{3}H-thymidine leaking from the GALT. However, the radioautographs of the lymphatic organs of both the intra-GALT and the i.p. labelled group contained considerable numbers of intensely labelled cells. An apparent overlapping of locally labelled and leakage labelled cell populations in terms of labelling intensities was thus noted, although labelled cells appeared more frequent in the intra-GALT labelled group. A special procedure was therefore described which defines a minimal labelling intensity of cells in

the radioautographs (amount developed silver overlying a cell) above which the probability that the labelled cell must have been locally labelled in the GALT can be predicted with a known level of significance.

IV:2. General evidence for cell migration

Such heavily labelled cells (HLCs), i.e. probable immigrants from the locally labelled GALT, were significantly more frequent (number per microscopic view field) in the radioautographs of all examined lymphatic organs of the intra-GALT labelled than in the i.p. labelled animal group. The HLC frequencies 48 h after labelling were highest in the mesenteric lymph nodes followed in order by the pharyngeal tonsil, the not locally labelled proximal (5th) Peyer's patch, the popliteal lymph node and the spleen. These results suggest therefore a migration of cells from the locally labelled parts of the GALT both to lymphatic tissue within (Peyer's patch and pharyngeal tonsil) and outside the GALT.

IV:3. Migrant cell types

The majority of the immigrants in the respective lymphatic organs were recovered as small lymphocytes (69-94%), but significant numbers of heavily labelled medium-sized to large lymphocytes were also found, except in the spleen. Of the other cell types, significant numbers of heavily labelled plasma cells were noted in the popliteal and mesenteric lymph nodes, marginal zone cells only in the spleen, reticular cells in the mesenteric lymph nodes and, finally, heavily labelled pyknotic cells in the lymph nodes and the Peyer's patch.

These results indicate that the immigrants in the respective lymphatic organs have this cytology 48 h after their local labelling in the GALT but do not allow any conclusion as to the cytological characteristic of the original emigrants from the locally labelled GALT.

IV:4. Localization of immigrants within lymphatic organs

The immigrant HLCs were in the radioautographs demonstrated in almost all morphologically defined parts of the respective target lymphatic organs. Some structures contained more HLCs than others, but for details of the extensive data on the localization patterns of the HLCs the reader is referred to Chapter IV. In general, immigrants were found in significant numbers both in structures regarded as thymus-independent (mean of 61% of all HLCs) and

thymus-dependent (24%). This makes it likely that both T and B lymphocytes participate in the cell migration from the GALT, with a probable dominance for the latter cell type.

IV:5. Labelling intensities of immigrants

The labelling intensity of HLCs in the lymphatic tissues was also studied. Details of the results are given in Chapter IV. One pertinent observation was that the labelling intensity appeared higher for HLCs localized in the thymus-dependent structures, e.g. the periarteriolar region in the spleen, than in the remaining parts of each tissue. Another finding was an increased labelling intensity for HLCs accumulated in the cortex near the cortico-medullary border of the lymph nodes. Such increased labelling intensity may be associated with recent immigrants in regions of cell traffic and/or with longlived lymphocytes.

CONCLUDING REMARKS

The present study thus revealed a migration of cells from the gut-associated lymphatic tissue (GALT) in young rabbits. Many of these migrants were B lymphocytes. The experimental approach and the results obtained could neither conclusively prove nor disprove a central, bursa equivalent, function in the studied parts of the GALT. However, the results provide a basis for further analysis of the nature of these GALT-derived B lymphocytes.

The combination of radioautography and precise morphological localization and cytological identification of labelled immigrants in lymphatic tissue should be conducive to such more specific studies of GALT-derived cells and to more general analyses of the traffic of T and B lymphocytes and their subpopulations between and within lymphatic tissues.

ACKNOWLEDGEMENTS

These investigations were carried out at the Department of Histology, Institute of Human Anatomy, at the University of Uppsala.

I wish to express my sincere thanks to:

Prof. KARL-ERIK FICHTELIUS, former head of the Department, who introduced me into the studies of the lymphocyte and lymphoid cell traffic. I thank him for generously placing research facilities at my disposal and for his advice and interest in my work.

Prof. T. JUHANI LINNA, now at the Department of Immunology and Microbiology, Temple University, Philadelphia, U.S.A., who has collaborated in part of this work. He has always shown a deep interest in my work and generously given valuable advice.

Assist. Prof. GUNNAR V. ALM for most valuable help, stimulating discussions and constructive criticism of the manuscript.

Prof. SVEN E. BROLIN, head of the Department for placing all the necessary resources at my disposal during the final stages of my work. His most kind interest and his never failing support was essential for the progress of my work.

Assoc. Prof. CLAES HELLERSTRÖM and Assoc. Prof. BIRGER PETERSSON for stimulating discussions and constructive examination of the manuscript.

Prof. GUNNAR EKLUND for general interest in my work and most valuable statistical advice.

All the members of the Royal Lymphatic Society of Uppsala and my colleagues at the Department of Histology, particularly Drs. GUNNAR TUFVESON, ROGER JUHLIN, INGER RIESENFELD and JAN SÄLLSTRÖM for all their support during this investigation.

Miss ANNSI HOLST, Mrs. RHODA LINNA, Mrs. BERIT ANDERSSON, Mrs BIRGITTA KULLGREN and Mrs. LILI-ANNE ERIKSSON for skilful technical assistance.

Mr. LARS PETTERSSON and Mr. ERIK JOHANSSON for excellent technical work.

Mrs. INGRID SWEDBERG, Mrs. KERSTIN CLAESSON, Mrs. LILA SINGH and Mrs. LIGORINA VAZ for excellent typing of the manuscripts and Mr. THORE WAHLUND for kindly preparing photographic details.

The staff of the Department for their supporting friendship.

The family ROSÉN who kindly provided me with excellent rabbits and advice on animal breeding.

GUNILLA, my wife, without you this work would never have been possible.

These investigations were supported by grants from the Swedish Medical Research Council and the Medical Faculty of the University of Uppsala.

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REKLAM & KATALOGTRYCK UPPSALA 1975

ISBN-91-506-0029-X